DEVELOPMENTAL CHANGES IN RESISTANCE OF MAMMALIAN EMBRYOS TO ELEVATED TEMPERATURE AND STRATEGIES TO IMPROVE FERTILITY IN DAIRY CATTLE DURING HEAT STRESS

By

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ABBREVIATIONS

ABAM	Antibiotic-antimycotic solution
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate hydroxylases
BRLC	Buffalo rat liver cells
BSA	Bovine serum albumin
BSO	D,L-buthionine-[S,R]-sulfoximine
BSS	Bovine steer serum
C	Centigrade
d	Day
DAPI	4',6'-Diamidino-2-phenylindole
DIM	Days in milk
DNA	Deoxyribonucleic acid
DNAk	Bacterial counterpart to mammalian HSC70
DPBS	Dulbecco's phosphate buffered saline
ETOH	Ethanol
FSH-P	Follicle-stimulating hormone (pituitary-derived)
g	Gram
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
h	Hour
hCG	Human chorionic gonadotrophin
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid radical
HSE	Heat shock elements
HSC	Heat shock protein cognate
HSF	Heat shock transcription factors
HSP	Heat shock protein
htFCS	Heat-treated fetal calf serum
IFNτ	Interferon-tau
IU	International unit
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
kDa	Kilodaltons
l	Liter

LH	Luteinizing hormone
m	Meters
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mo	Month
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
$\cdot O_{2^-}$	Superoxide radical
$\cdot OH$	Hydroxyl radical
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
pI	Isoelectric point
PMSG	Pregnant mare serum gonadotropin
$pp60^{src}$	Rous sarcoma virus transforming protein
R_2O_2	Peroxide residue
ROH	Reduced peroxide; stable hydroxyl
SEM	Standard error of the mean
TALP	Modified Tyrode's solutions
v/v	Volume/volume
wk	Week
w/v	Weight/volume
yr	Year

Abstract of Dissertation Presented to the Graduate School
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DEVELOPMENTAL CHANGES IN RESISTANCE OF MAMMALIAN EMBRYOS TO ELEVATED TEMPERATURE AND STRATEGIES TO IMPROVE FERTILITY IN DAIRY CATTLE DURING HEAT STRESS

BY

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Embryos gain resistance to adverse effects of heat stress as development progresses. The primary goals of this dissertation research were to identify the ontogeny of thermal resistance in mammalian embryos and identify systems to limit effects of heat on bovine embryo survival. In the first study, embryonic survival was decreased in cows heat-stressed on d 1 of pregnancy but not on d 3, 5 or 7 of pregnancy, implying that embryos gain increased thermal resistance by d 3 of pregnancy. The developmental resistance of embryos to heat was verified by exposure of bovine embryos to elevated culture temperatures (*i.e.,* heat shock). Subsequent *in vitro* development was decreased for 2-cell bovine embryos but not for bovine morulae exposed to 41 C for 3 h. The acquisition of intracellular processes

which confer thermal resistance was investigated by determining the ontogeny of induced thermotolerance in murine embryos (*i.e.,* ability of embryos to resist a severe heat shock by prior exposure to a mild heat shock). Induction of thermotolerance was observed between the 8-cell and blastocyst stage for murine embryos, suggesting that intracellular thermoprotective mechanisms are acquired during embryonic development.

Two strategies for limiting effects of heat stress on bovine embryos were examined. Pregnancy rates were slightly improved in cows exposed to short-term cooling from 2 to 3 d before until 5 to 6 d after breeding. Two molecules, glutathione and taurine, reduced heat shock effects on viability and/or development of cultured bovine and murine morulae but did not reduce heat shock effects on cultured 2-cell bovine embryos. In conclusion, embryos gain resistance to elevated temperatures as development progresses, probably through acquisition of intracellular thermoprotective mechanisms. Pregnancy rates were improved in cows by short-term cooling. However, glutathione and taurine could not protect embryos during initial stages of development from the adverse effects of elevated temperature.

CHAPTER I
INTRODUCTION

Failure of reproduction is costly to the American livestock industry; in 1978, reproduction-associated losses for farm animals were estimated at $1.4 billion (Gerrits *et al.,* 1979). In lactating dairy cows, it has been estimated that $2.37 to $4.63 of gross income is lost each day a cow remains non-pregnant after 90 days postpartum (Ferris and Fogwell, 1984). One phenomenon that contributes to economic losses in cattle is heat-stress induced infertility. Pregnancy rates of lactating cows in Arizona, Florida and Israel have been reported to decrease from 40 to 60% during winter months to as low as 10 to 20% during summer months (Stott and Williams, 1962; Badinga *et al.,* 1985; Berman and Wolfenson, 1992). Elevated ambient temperatures also have been reported to reduce pregnancy rates in mice (Elliot *et al.,* 1968; Elliot and Ulberg, 1971; Bellve, 1972; Baumgartner and Chrisman, 1981a; Baumgartner and Chrisman, 1981b), rabbits (Ulberg and Sheean, 1973; Wolfenson and Blum, 1988), pigs (Warnick *et al.,* 1965; Tompkins *et al.,* 1967; Edwards *et al.,* 1968; Omtvedt *et al.,* 1971), and sheep (Dutt *et al.,* 1959; Dutt, 1963; Woody and Ulberg, 1964). Thus, the problem that heat stress poses to the female is of general importance to mammals.

The effects of heat stress in cattle can be avoided by provision of intensive cooling systems for the first 150 to 200 d of lactation. Pregnancy rates were

increased to rates observed during winter months with exposure to repetitive sprinkling/forced ventilation (Wolfenson *et al.,* 1988) and air-conditioning (Stott *et al.,* 1972; Thatcher *et al.,* 1974). However, since the economic gain from increased fertility and milk yield may not be sufficient to make systems such as these feasible on commercial operations, additional approaches must be investigated. The maturing oocyte and early developing embryo appear to be highly susceptible to heat stress effects in cattle (Dunlap and Vincent, 1971; Putney *et al.,* 1988a; Putney *et al.,* 1989a). It is quite possible that if periods of oocyte and embryonic sensitivity to heat stress and biochemical processes which promote thermal resistance in embryos are investigated, additional methods may be available to limit heat stress effects on pregnancy rates.

Primary goals of this dissertation research were to determine when embryos gain increased resistance to heat stress effects and identify systems to prevent heat stress effects. Identification of the ontogeny of thermal resistance was accomplished for bovine embryos *in vivo* and for bovine and murine embryos *in vitro*. Studies completed *in vitro* with murine embryos were also used to evaluate whether the ontogeny of thermal resistance is correlated with previous reports of the ontogeny of heat shock proteins (HSPs) which protect intracellular macromolecules from heat. Potential methods for reducing heat stress effects on early embryonic development were examined. The first was maintaining cows in a cooled environment around the time of breeding and early embryonic development. Secondly, use of antioxidants as thermoprotectants for cultured embryos was pursued in this dissertation.

CHAPTER II
REVIEW OF LITERATURE

Effects of Elevated Temperature on Reproduction in Dairy Cattle

Summer infertility in dairy cattle is caused by the combination of decreased estrous detection (Gangwar *et al.,* 1965; Roller and Stombaugh, 1974; Wolff and Monty, 1974; De Silva *et al.,* 1981; Gwazdauskas *et al.,* 1981; Thatcher and Collier, 1986; Wolfenson *et al.,* 1988) and lowered pregnancy rates (Erb *et al.,* 1940; Stott, 1960; Poston *et al.,* 1962; Stott and Williams, 1962; Dunlap and Vincent, 1971; Ingraham *et al.,* 1974; Badinga *et al.,* 1985; Cavestany *et al.,* 1985; Monty and Wolff, 1974; Udomprasert and Williamson, 1987; Putney *et al.,* 1988a; Putney *et al.,* 1989a). Environmental conditions such as elevated ambient temperature, high humidity and intense solar radiation produce a stress on the animal, termed heat stress, which disrupts the animal's ability to maintain a balance between heat production and heat loss. The resulting hyperthermia and the physiological changes induced to limit hyperthermia can have detrimental effects on reproductive processes such as estrous detection, fertilization, embryonic development, fetal development, uterine function and changes in hormonal patterns. This review will focus on describing effects of heat stress on reproductive processes and physiological and biochemical mechanisms to limit these effects. Heat stress is detrimental to establishment of pregnancy in

mice (Elliot *et al.,* 1968; Elliot and Ulberg, 1971; Bellvé, 1972; Baumgartner and Chrisman, 1981a; Baumgartner and Chrisman, 1981b), rabbits (Ulberg and Sheean, 1973; Wolfenson and Blum, 1988), pigs (Warnick *et al.,* 1965; Tompkins *et al.,* 1967; Edwards *et al.,* 1968; Omtvedt *et al.,* 1971), and sheep (Dutt *et al.,* 1959; Dutt, 1963; Woody and Ulberg, 1964). Since processes by which heat stress alters fertility in cattle are probably similar to heat-stress induced infertility in other species, information from these species will be used when illustrative of mechanisms that are occur in cattle.

Estrous Detection

Failure of estrous detection is a primary cause for decreased reproductive performance in dairy cattle under many conditions and is amplified by heat stress. In one study (Thatcher and Collier, 1986), 66% of potential heats were undetected in a commercial herd under thermoneutral conditions while approximately 80% of potential heats were not detected during heat stress (Thatcher and Collier, 1986). The decrease in estrous detection during heat stress is a result of decreased duration and intensity of estrous activity. During periods of heat stress, the duration of estrus can be reduced to as low as 8 h in lactating cows (Wolff and Monty, 1974; Gwazdauskas *et al.,* 1981; Wolfenson *et al.,* 1988) and the number of mountings during estrus can be reduced dramatically (Roller and Stombaugh, 1974; De Silva *et al.,* 1981).

Although causes for diminished estrous activity during heat stress have not been determined, two feasible explanations exist. One possibility is that the degree

of behavioral estrus is reduced to decrease metabolic production associated with physical activity. Alternatively, physiological events that influence estrous behavior can be altered by heat stress. In this regard, periovulatory surges of estradiol are diminished in heat-stressed cattle (Gwazdauskas *et al.,* 1981; Roman-Ponce *et al.,* 1981) and pigs (Flowers and Day, 1990). Attenuated estradiol secretion at estrus may be caused by decreased preovulatory or basal concentrations of luteinizing hormone (LH; Wise *et al.,* 1988; Younas *et al.,* 1993) or decreased follicle growth (Badinga *et al.* 1993) during heat stress.

Fertilization

Fertilization is usually not a major contributing factor to infertility in cattle. Fertilization rates in cattle maintained in thermoneutral environments and bred by either natural breeding or artificial insemination have been estimated to be 80 to 100% (Kidder *et al.,* 1954). Furthermore, rate of fertilization was unaffected by heat stress in heifers (Putney *et al.,* 1989a), sheep (Dutt, 1963), mice (Elliot and Ulberg, 1971; Baumgartner and Chrisman, 1988) and rabbits (Alliston *et al.,* 1965). Other reports, however, have documented that maternal heat stress of ewes decreased fertilization rates (Dutt *et al.,* 1959; Alliston *et al.,* 1961; Alliston and Ulberg, 1961). Fertilization of cultured bovine oocytes was decreased by 24 h exposure to 41 C but not to 40 C (Lenz *et al.,* 1983).

There are other deleterious consequences of heat stress during the period accompanying fertilization. In particular, embryonic development and survival rates for cattle (Putney *et al.,* 1989a), sheep (Dutt, 1963; Woody and Ulberg, 1964), mice

(Elliot *et al.,* 1968; Elliot and Ulberg, 1971; Baumgartner and Chrisman, 1988) and rabbits (Alliston *et al.,* 1965) were reduced by exposure of animals to heat stress during either fertilization or the period of final oocyte maturation preceding deposition of spermatozoa. The above mentioned experiments suggest that heat stress can disrupt the periovulatory oocyte or spermatozoa in a manner that results in formation of an embryo with reduced competence.

Effects on oocytes. In mice, maternal heat stress during final oocyte maturation caused disruption of spindle fiber formation during metaphase I and increased the incidence of oocytes that did not undergo normal meiosis (Baumgartner and Chrisman, 1981a; Baumgartner and Chrisman, 1981b). Other investigators have observed that maternal heat stress during this period caused oocyte degeneration, polar body retention and increased incidence of aneuploidy (Branden and Austin, 1954). Parthenogenic activation has also been observed in heat-shocked mouse oocytes (Komar, 1973; Balhkier and Tarkowski, 1976). A possible cause for oocyte sensitivity to elevated temperatures is a lack of intracellular protective mechanisms such as heat-shock proteins (Manejwala *et al.,* 1991). This subject will be pursued in more detail in later sections.

Maternal heat stress may also alter oocyte quality through disruption of hormonal regulation of oocyte maturation and ovulation. Preovulatory surges of LH (Younas *et al.,* 1993) and estradiol (Gwazdauskas *et al.,* 1981; Roman-Ponce *et al.,* 1981) are decreased during heat stress in cattle and this could affect initiation and timing of ovulation. Secondly, follicle development may also be affected adversely

by heat stress. Serum concentrations of LH have been reported to be decreased throughout the estrous cycle of heat-stressed cows (Wise *et al.,* 1988) and this may be a cause for diminished follicle growth during periods of heat stress (Badinga *et al.* 1993).

Effects on spermatozoa. Sensitivity of spermatozoa to elevated temperatures in the female reproductive tract also contributes to decreased fertility. Incubation of rabbit spermatozoa at 40 C for 3 h had no deleterious effects on *in vivo* fertilization rate but subsequent embryonic survival was decreased (Burfening and Ulberg, 1968). Similarly, fertilization rate of female rabbits maintained at thermoneutral conditions were unaffected by insemination with spermatozoa that had been recovered from a mated female that was exposed to heat stress but, again, subsequent embryonic development was decreased (Howarth *et al.,* 1965). Monterroso *et al.* (1994) reported that exposure of spermatozoa to 41 or 42 C for 3 h did not effect cleavage rate of bovine oocytes *in vitro* but subsequent development was impaired. Perhaps elevated temperatures promote chromosomal damage of spermatozoa, similar to that of oocytes in a way that affects embryonic development. The validity of this hypothesis may be argued, however, because spermatozoa do not undergo meiotic events during their residence in the reproductive tract and spermatozonal chromosomes are tightly packaged in cattle and other species (Amann and Schanbacher, 1983). Also, Monterroso *et al.* (1994) found no effect of heat shock on DNA damage of bovine spermatozoa using acridine orange as an indicator.

Effects on spermatogenesis. Adverse effects of heat stress on bulls (Erb *et al.,* 1942) can contribute to summer infertility in cattle bred by natural breeding. Heat-stressed bulls have semen with reduced motility, concentration and total amount of spermatozoa per ejaculate (Casady *et al.,* 1953; Johnson and Branton, 1953; Branton *et al.,* 1956; Fryer *et al.,* 1958). These effects are caused by a disruption of spermatogenesis. Rates of fertilization and implantation, and fetal survival were decreased for 30 d following exposure of male mice to heat stress (Burfening *et al.,* 1970). Similarly, sperm quality is decreased for 6 to 10 wk following exposure of bulls to heat stress (Casady *et al.,* 1953; Skinner and Louw, 1966; Meyerhoeffer *et al.,* 1985). This period of decreased sperm quality coincides with the duration of spermatogenesis (45 to 60 d; Amann and Schanbacher, 1983). Spermatocytes, spermatids and B spermatogonia appear to be the most sensitive cells to elevated temperatures (DeAlba and Riera, 1966; Amann and Schanbacher, 1983).

Early Embryonic Development

Several lines of evidence indicate that embryonic death is increased by heat stress. As previously mentioned, exposure to heat stress during oocyte maturation and fertilization causes decreased embryonic development in several species (Dutt, 1963; Woody and Ulberg, 1964; Alliston *et al.,* 1965; Elliot *et al.,* 1968; Elliot and Ulberg, 1971; Baumgartner and Chrisman, 1988; Putney *et al.,* 1989a). Embryonic survival is also decreased by exposure of cattle to heat stress during the first 3 d (Dunlap and Vincent, 1971) or 7 d (Putney *et al.,* 1988a) following breeding. Monty and Racowsky (1987) determined that embryos collected 6 to 8 d following

fertilization had a higher incidence of death during summer months than winter months. Observations in sheep (Alliston and Ulberg, 1961; Dutt, 1963), swine (Tompkins *et al.,* 1967; Wettemann *et al.,* 1988), mice (Bellvé, 1976) and rabbits (Wolfenson and Blum, 1988) also indicate that maternal heat stress during the first few days of pregnancy causes a large increase in embryonic mortality.

Embryos become more resistant to adverse effects of maternal heat stress as development proceeds. In swine, exposure to heat stress during the first 5 to 8 d of pregnancy decreased subsequent embryonic survival, whereas heat stress after this period did not alter embryonic survival rates (Tompkins *et al.,* 1967; Edwards *et al.,* 1968; Omtvedt *et al.,* 1971). The ontogeny of embryonic resistance to heat stress was further defined in sheep. Embryonic viability was decreased with maternal heat stress on either d 0 (breeding), 1, 3, 5, or 7 of pregnancy (Dutt, 1963). However, when comparing heat stress effects among days, heat stress on d 0 and 1 of pregnancy caused more severe embryonic losses than heat stress on d 3, 5 or 7 of pregnancy (Dutt, 1963). Similarly, exposure of rabbit embryos to 40 C for 6 h in culture was detrimental to subsequent *in vivo* development at the 1-cell stage but not at the 2-cell stage (Alliston *et al.,* 1965). Cultured murine 1-cell embryos were also more sensitive to heat shock than 2-cell embryos (Gwasdauskas *et al.,* 1992).

It has not been determined whether a similar phenomenon occurs in cattle. Indirect evidence, however, suggests that bovine embryos gain resistance to heat stress effects during early stages of development. There is a negative correlation of environmental and uterine temperature with pregnancy rate on the day of

insemination or the day following insemination in dairy cattle (Ingraham *et al.,* 1974; Gwasdauskas *et al.,* 1975; Badinga *et al.,* 1985). Biggers *et al.* (1987) found no effect of heat stress from d 8 to 16 of pregnancy on pregnancy rate but embryonic development was decreased. In another study (Putney *et al.,* 1989b), transfer of d 7 embryos retrieved from superovulated heifers to heat-stressed lactating dairy cows increased pregnancy rates (29.2%) as compared with pregnancy rates of cows which were inseminated artificially (13.5%). Similarly, there was no seasonal variation in pregnancy rates resulting from transfer of frozen/thawed embryos to recipients located in the Southwest United States (Putney *et al.,* 1988c).

There are two possible mechanisms by which heat stress could disrupt embryonic development. First, elevated body temperatures could directly alter rate of embryonic development through influences of heat on cell survival. Alternatively, heat stress could act indirectly, by modifying functions of the uterus and oviducts. Alliston and Ulberg (1961) conducted a study using reciprocal embryo transfer in sheep to determine the relative contribution of these two mechanisms. The pregnancy rate for embryos transferred at d 3 from a heat-stressed donor to a nonstressed recipient were 9.5%. Pregnancy rate of embryos transferred from a nonstressed donor to a heat-stressed recipient were 24.0%. In contrast, pregnancy rates for embryos transferred from a thermoneutral donor to a nonstressed recipient were 56.5%. Therefore, both direct and indirect effects were involved with the heat-induced decrease in embryonic survival, with direct effects of heat being more deleterious. Other evidence for a direct effect of heat on embryonic survival is

provided from studies in which exposure of embryos to elevated culture temperatures, or heat shock, reduced embryonic survival. For example, Alliston *et al.* (1965) found that exposure of 1-cell rabbit embryos to 40 C for 6 h in culture decreased subsequent embryonic survival *in utero*. In vitro development was also decreased in mouse embryos exposed to 39 C for 96 h (Gwasdauskas *et al.,* 1992) or 42 to 43 C for 2 h (Aréchiga *et al.,* 1992; Malayer *et al.,* 1992; Aréchiga *et al.,* 1994a).

Uterus and Oviduct

The uterus and oviduct are responsible for secreting nutrients, growth regulators and other agents which promote establishment and maintenance of pregnancy. Hence, alterations in the function of the uterus and oviduct by heat stress could be detrimental for fertilization, embryonic development and maintenance of pregnancy. As mentioned previously, a reciprocal embryo transfer study indicates that substantial decreases in pregnancy rate result from effects of heat stress on the uterus (Alliston and Ulberg, 1961).

Detrimental effects of heat stress on the uterus and oviduct may be caused, in part, by altered synthesis and secretion of uterine and oviductal proteins in direct response to elevated temperatures. Culture of endometrial explants from cows at 43 C increased protein synthesis and secretion by the endometrium (Malayer *et al.,* 1988; Malayer and Hansen, 1990). In one study (Malayer *et al.,* 1988), secretion of seven individual proteins from the ipsilateral uterine horn of cows (obtained from d 0 to d 8 postestrus) were decreased by exposure to heat shock. In a second study (Malayer and Hansen, 1990), heat shock decreased synthesis and secretion of

proteins from oviductal explants ipsilateral to the site of ovulation for Holstein cows. Malayer and Hansen (1990) also determined that in contrast to protein secretion from oviducts of Holsteins, heat shock increased protein secretion from oviductal explants ipsilateral to the site of ovulation for Brahman cows. Perhaps these differences are related to enhanced thermal tolerance of Brahmans to adverse effects of heat stress on reproduction. At d 17 of pregnancy, *in vitro* exposure to elevated temperatures did not affect endometrial explant synthesis or secretion of proteins (Putney *et al.,* 1988b), whereas *in vivo*, uterine content of proteins collected from the ipsilateral uterine horn at d 16 were decreased with heat stress from d 8 to 16 (Geisert *et al.,* 1988).

Alterations in protein synthesis and secretion by the uterus and oviduct may also be caused by altered secretion of progesterone and estradiol. Several studies have reported decreased serum progesterone concentrations during summer months (Rosenberg *et al.,* 1977; Wise *et al.,* 1988; Wolfenson *et al.,* 1988; Imtiaz-Hussain *et al.,* 1992; Younas *et al.,* 1993; Howell *et al.,* 1994). In contrast, increased plasma concentrations of progesterone have also been reported during heat stress (Abilay *et al.,* 1975; Vaught *et al.,* 1977; Roman-Ponce *et al.,* 1981). This latter effect is probably short-lived and caused by a possible acute heat stress effect on adrenal secretion of progesterone (Thatcher, 1974). Roman-Ponce *et al.* (1981) and Gwasdauskas *et al.* (1981) also concluded that acute heat stress decreased the peri-ovulatory rise in serum estradiol concentrations. This effect is probably caused by reduced follicular development during heat stress (Badinga *et al.,* 1993). Alterations

in uterine blood flow may also influence uterine and oviductal function. Estradiol-stimulated uterine blood flow was reduced in cows exposed to heat stress (Roman-Ponce *et al.,* 1978).

Regulation of Luteal Function by the Uterus and Embryo

Conceptus tissue protein content and protein secretion at d 17 of pregnancy is affected by heat stress. Exposure of conceptuses to 43 C for 18 h decreased overall protein synthesis and secretion and decreased secretion of interferon-τ (IFNτ; Putney *et al.,* 1988b). In contrast, secretion of IFNτ from conceptuses was unaffected by maternal heat stress from d 8 to 16 of pregnancy (Geisert *et al.,* 1988). Secretion of $PGF_{2\alpha}$ from the endometrium is altered during periods of thermal stress. Basal secretion of $PGF_{2\alpha}$ from endometrial explants at d 17 of pregnancy or d 17 of the cycle was increased by heat shock (Putney *et al.,* 1988b; Putney *et al.,* 1988c; Putney *et al.,* 1989c; Malayer *et al.,* 1990). *In vivo*, heat stress of cyclic cows on d 17 postestrus increased oxytocin-stimulated release of $PGF_{2\alpha}$ in one study (Putney *et al.,* 1989c) but not another (Wolfenson *et al.,* 1993). Basal secretion of $PGF_{2\alpha}$ was also increased in pregnant cows exposed to heat stress (Putney *et al.,* 1989c). Under thermoneutral conditions, oxytocin-stimulated release of $PGF_{2\alpha}$ from endometrium on d 17 of pregnancy is minimal (Putney *et al.,* 1989c; Wolfenson *et al.,* 1993). In one study (Wolfenson *et al.,* 1993), heat stress caused increased levels of oxytocin-stimulated $PGF_{2\alpha}$ for pregnant cows. In another study (Putney *et al.,* 1989c), oxytocin-stimulated release of $PGF_{2\alpha}$ was unaffected by maternal heat stress, although few pregnant cows were used in this study. Oxytocin-stimulated $PGF_{2\alpha}$

release also was increased from endometrial explants of pregnant cows exposed to heat shock (Putney *et al.,* 1988b). Taken together, these results suggest that both basal and oxytocin-stimulated secretion of $PGF_{2\alpha}$ is increased for both cyclic and pregnant cows exposed to heat stress.

Fetal Development

Exposure of animals to heat stress during mid and late gestation can promote fetal malformations in several species (Mirkes, 1987; Webster *et al.,* 1985; Tikkanen and Heinonen, 1991). Imposition of heat stress at d 9 to 10 of pregnancy can increase the incidence of cranial malformations such as microphthalmia, encephalocele and maxillary hypoplasia in rats (Webster *et al.,* 1985). This effect is caused by a direct action of elevated temperature on the fetus since developmental defects have also been observed after exposure of cultured d 9 rat fetuses to heat shock (Mirkes, 1987). Fever in pregnant women has been reported to increase the incidence of cardiovascular malformations in offspring (Tikkanen and Heinonen, 1991).

Since approximately 60% of fetal growth occurs during the last 90 d of gestation in cattle (Eley *et al.,* 1978), hyperthermia could be detrimental to fetal development during this period. Heat stress during the last few months of pregnancy causes decreased birth weights of dairy calves (Bonsma, 1949; Collier *et al.,* 1982; Thatcher and Collier, 1982) and decreased placental tissue weights (Head *et al.,* 1981). Heat stress also affects other species in a similar manner including mice (Pennicuik, 1966), rats (Arora *et al.,* 1979) and sheep (Alexander and Williams, 1971;

Brown *et al.,* 1977; Brown and Harrison, 1981; McCrabb *et al.,* 1993). The decrease in birth weight is probably a consequence of decreased uterine and placental blood flow, as evidenced in sheep (Alexander and Williams, 1971; Brown *et al.,* 1977; Brown and Harrison, 1981; McCrabb *et al.,* 1993). In heat-stressed cattle, decreased birth weights are associated with decreased prepartum concentrations of estrone sulfate in maternal serum (Collier *et al.,* 1982), confirming that placental function is impaired by heat stress. Collier *et al.* (1982) also found that exposure of cows to heat stress during the last 90 d of gestation reduced subsequent milk yield. This effect is probably due to decreased lactogenesis caused by decreased placental function (Thatcher *et al.,* 1980; Collier *et al.,* 1982). In contrast, traits such as days to first estrus, days open and services per conception were not affected by exposure to heat stress during late gestation (Lewis *et al.,* 1984).

Mechanisms by Which Cells are Protected from Effects of Elevated Temperature

Mechanisms of Heat Induced Cell Damage

Elevated body temperatures and exposure of cells to elevated culture temperatures (*i.e.,* heat shock) adversely affect structure and functional capabilities of lipids, proteins and nucleic acids. Alterations of cellular components by heat shock occurs as a result of three potential mechanisms. First, elevated temperature can directly affect structure and function of macromolecules and cellular organelles. Secondly, cellular production of free radical molecules can be increased by heat

shock and these molecules can in turn oxidize most cellular molecules. Lastly, heat shock may induce the onset of programmed cell death, or apoptosis.

Direct effects of heat shock. Lipids in cellular membranes are highly sensitive to changes in temperature. Increased temperatures of 40 to 45 C causes bilayer expansion and formation of inverted micelles (Overath *et al.,* 1970; McElhaney, 1974; Yatvin, 1977; Bowler, 1981). These conformational changes, termed membrane blebbing, are caused primarily by decreased stability of saturated fatty acids (Konings, 1988). One consequence of increased fluidity of membranes following heat shock is alterations in ion permeability. For example, calcium influx increases during heat shock (Stevenson *et al.,* 1986; Stevenson *et al.,* 1987), and this can promote cell death in several cell types (Fawthrop *et al.,* 1991).

Protein denaturation also occurs upon exposure to temperatures of 40 to 45 C, primarily through disruption of weak amino acid bonds that confer tertiary and quartenary structure (*i.e.,* hydrogen bonds and hydrophobic interactions; Privalov, 1979; Lepock *et al.,* 1983). Heat-induced alterations in protein structure cause disassembly of microtubules (Coss *et al.,* 1982; Wiegant *et al.,* 1987), intermediate filaments (Wiegant *et al.,* 1987; Welch and Mizzen, 1988) and microfilaments (Welch and Suhan, 1985; Iida *et al.,* 1986). As a result, cell division, cell-cell communication, and cell adherence to substrates can be impaired. It has also been shown that conformational changes in membrane-associated proteins caused by heat shock can alter signal transduction systems (Lepock *et al.,* 1983; Calderwood *et al.,* 1985; Lin and Hahn, 1988). Not all proteins undergo reduced function at temperatures of 40

to 45 C, however (Ashburner and Bonner, 1979; Walton *et al.,* 1989). For example, activity of ornithine decarboxylase was maximal at 40 C in rat tissues, whereas activity decreased dramatically with exposure to higher temperatures (Penafiel *et al.,* 1988). This biphasic effect is probably caused by an increase in specific activity of enzymes at slight elevations in temperature but denaturation of enzymes as a result of more severe heat shock.

Purine and pyrimidine ring structures on nucleic acids are also sensitive to heat shock. Modifications in ring structures have been observed to cause unwinding of supercoiled DNA, double stranded DNA breaks, chromosomal exchanges and increased incidence of aneuploidy (Vig, 1979; Sherwood *et al.,* 1987; Mackey *et al.,* 1988; Borrelli *et al.,* 1991). Temperatures required to produce such effects are high (>45 C), however, suggesting that this effect is not observed during exposure of most cells to elevated temperatures. Additionally, repair of damaged DNA is retarded following exposure to heat shock, since activities of topoisomerase enzymes (particularly topoisomerase II) are reduced by heat shock (Dewey and Esch, 1982; Chu and Dewey, 1987). Translational capabilities of cells also are affected adversely by heat shock because of alterations in ribosomal RNA ring structure and denaturation of ribosomal-associated proteins (Warocquier and Scherrer, 1969; Bouche *et al.,* 1979; Sadis *et al.,* 1988).

Free radicals. In addition to direct actions of heat shock on cellular components, lipids, proteins and nucleic acids also may be modified by reaction with reduced oxygen species. Such molecules include free radicals, which contain an

unpaired electron, as well as other reactive oxygen species (*i.e.*, H_2O_2). Production of free radicals (Skibba *et al.*, 1986; Freeman *et al.*, 1990; Lin *et al.*, 1991) and intracellular antioxidant systems (Omar and Lanks, 1984; Loven *et al.*, 1985; Harris *et al.*, 1991) have been shown to increase during heat shock.

Free radicals are formed by normal metabolism of oxygen by mitochondrial electron transport chain reactions and metabolic reactions in the endoplasmic reticulum and peroxisome. Normally, four electrons are transferred to oxygen to yield water ($O_2 + 4e^- \dashrightarrow 2\ H_2O$; Chance *et al.*, 1979) and generate ion gradients for phosphorylation of desired substrates. However, oxygen also readily accepts single electron transfers to yield formation of superoxide ($\bullet O_2^-$) by univalent electron acceptance, hydrogen peroxide (H_2O_2) by bivalent electron acceptance, and hydroxyl radical ($\bullet OH$) by trivalent electron acceptance (Fidovich, 1975; Chance *et al.*, 1979; Allen, 1991). The most prevalent free radical formed by mitochondrial electron transfer is $\bullet O_2^-$ (Ramasarma, 1982; DiGuiseppi and Fridovich, 1984), which can be readily dismutated to H_2O_2 by superoxide dismutase (Chance *et al.*, 1979). The $\bullet OH$ radical is formed primarily by reaction of $\bullet O_2^-$ and H_2O_2 with heavy metals or with each other (Halliwell and Gutteridge, 1984). Additionally, free radicals are formed by metabolism of specific cellular molecules such as xanthine and hypoxanthine (Halliwell, 1987). It is possible that during heat shock, disruption of electron transport systems causes increased production of free radicals.

Immediately following formation, free radicals react with cellular molecules for abstraction of hydrogen, addition to aromatic ring structures, or electron transfer

(Halliwell and Gutteridge, 1984). However, $\cdot OH$ and $\cdot O_2^-$ radicals are more reactive than H_2O_2. The most devastating effect of free radical reactivity on cells is peroxidation of membranes caused by extraction of hydrogen molecules from lipids. The reactive carbon molecule formed by reaction with free radicals forms a peroxide which extracts hydrogen from other fatty acids, thus catalyzing continuous peroxidation of lipids (Sevanian and Hochstein, 1985; Halliwell, 1987). The resulting peroxidation decreases membrane stability, alters ion and macromolecule transport, and increases production of water soluble aldehydes which can cause membranes to completely lose their integrity (Comporti, 1985; Sevanian and Hochstein, 1985). Additionally, free radicals react with thiol groups or hydrophobic residues of proteins (Wolff and Dean, 1986; Davies *et al.,* 1987; Hunt *et al.,* 1988; Pacifici *et al.,* 1989; Salo *et al.,* 1990) and purine and pyrimidine structures of nucleic acids (Johnson and Demple, 1988; Teebor *et al.,* 1988; Povirk and Steighner, 1989; Simic *et al.,* 1989).

Apoptosis. It is possible that heat shock promotes programmed cell death (apoptosis) in cells. Apoptosis represents cell death occurring from intrinsic cellular stimuli, which usually requires novel protein synthesis (Kerr *et al.,* 1972; Fawthrop *et al.,* 1991; Eastman, 1993). Such a phenomenon is observed during development (Gramzinski *et al.,* 1990) as well as in differentiated cells (Duvall and Wyllie, 1986). However, it remains unknown whether apoptosis is a mechanism by which heat shock causes cell death.

Induced thermotolerance. Although heat shock promotes damage of many cellular components, such effects may be limited through the presence of intracellular

mechanisms which confer thermal resistance. Such systems include HSPs, which serve to protect cellular components from potential damage caused by heat shock, and antioxidants, which scavenge free radicals and restore cellular components that have reacted with free radicals. Resistance of cells to heat shock can be further enhanced by induction of thermotolerance. This is a transient cellular event defined as the ability of cells to withstand a severe heat shock after prior exposure to a less severe heat shock (Gerner and Schneider, 1975; Henle and Leeper, 1976). Induction of thermotolerance has been observed to reduce effects of heat shock on survival of d 10 rat embryos (Mirkes, 1987), viability of several cell lines (Gerner and Schneider, 1975; Henle and Leeper, 1976; Li and Werb, 1982; Li and Mak, 1989; Maytin *et al.,* 1990; Hatayama *et al.,* 1991), translational activity of cells (Mizzen and Welch, 1988) and cytoskeletal disassembly (Welch and Mizzen, 1988). It is possible that embryonic survival could be improved by manipulation of these mechanisms if the regulation of thermal resistance or regulation of induced thermotolerance for embryos can be further defined during early embryonic development. The following sections will review mechanisms involved with conferring thermal resistance of cells.

Heat Shock Proteins

Heat shock induced gene expression is a ubiquitous phenomenon of eukaryotic and prokaryotic cells (Nover and Scharf, 1991). A major series of such heat-inducible proteins include heat shock proteins (HSPs). Since many HSPs serve as chaperone molecules in cells during non-stress conditions, HSPs have been implicated in limiting heat shock-induced cell damage through association with

cellular components. The protective actions of HSPs involve preventing structural damage of proteins and nucleic acids or targeting damaged components for degradation or rejuvenation. The protective role of HSPs are not limited to heat shock; synthesis of HSPs also occurs with exposure to other stresses including anoxia (Sciandra *et al.,* 1984), oxidizing agents (Kim *et al.,* 1983a; Spitz *et al.,* 1987), chelating agents (Levinson *et al.,* 1980), sulfhydryl agents (Levinson *et al.,* 1979; Carlson and Rechsteiner, 1987), amino acid analogs (Kelley and Schesinger, 1978; Li and Laszlo, 1985; Hatayama *et al.,* 1986), gene expression inhibitors (Hatayama *et al.,* 1986; Lee and Dewey, 1987), organic solvents (Hahn *et al.,* 1985), viral infection (Rose and Khandjian, 1985), mutagenic agents (Carr *et al.,* 1986; Forance *et al.,* 1989a), high pH (Whelan and Hightower, 1985), serum or mitogen supplementation (Wu and Morimoto, 1985; Wu *et al.,* 1986), glucose deprivation (Sciandra and Subjeck, 1983; Whelan and Hightower, 1985), ischemia (Cairo *et al.,* 1985) and ultraviolet radiation (Brunet and Giacomoni, 1989). The chaperone characteristics of HSPs are likely to also aid in protecting cells from damage caused by a number of these stress conditions as well as to play an essential role in normal cellular function.

Proteins within the HSP family are identified according to their molecular weight; for example, HSP70 represents heat-induced proteins whose molecular weight is approximately 70 kDa. HSPs have also been classified into distinct protein classes based on similarities in molecular weight, sequence homology and function. Six major families of HSPs are identified currently; HSP110, HSP90, HSP70, HSP60,

HSP27 and ubiquitin (Nover and Scharf, 1991; Welch, 1992). Genes encoding HSPs are highly conserved; at least 40% sequence homology has been observed between bacterial and human HSP genes (Craig, 1985; Lindquist, 1986). This high conservation suggests that these proteins are essential for cell function and survival during heat shock or other cellular stresses. Additionally, roles for many HSPs have been identified in cells under normal conditions and evolutionary conservation may also have been achieved because of essential constitutive roles for HSPs. The following section will review structure, function and gene regulation of HSPs and propose specific functions accomplished by HSPs during heat shock which confer cellular resistance to heat.

HSP70. Heat shock proteins within this class represent a major group of heat-induced proteins within most cells. Multiple genes encode a number of proteins in this class, which can be classified according to differential gene expression patterns (Nover and Scharf, 1991; Welch, 1992; McKay, 1993). One group of proteins includes heat shock cognate proteins, which are constitutively expressed proteins of 70 to 76 kDa with a pI of 5.3 to 5.6 (Dworniczak and Mirault, 1987; Sorger and Pelham, 1987; Giebel *et al.,* 1988; Masumi *et al.,* 1990). Cognate proteins, abbreviated as HSC70 in this dissertation, are enhanced 2 to 3 fold by heat shock (Milarski *et al.,* 1989). A second group of proteins, abbreviated as HSP68 in this dissertation, are highly heat-inducible proteins. Expression of HSP68 proteins is low during nonstressed conditions but increases 20 to 30 fold in response to heat shock (Milarski *et al.,* 1989). In comparison to HSC70, HSP68 proteins are slightly lower

in molecular weight (68 to 74 kDa) and of more basic pI (5.6 to 6.3; Lowe and Moran, 1984; Hunt and Calderwood, 1990; Masumi *et al.,* 1990; Meerson *et al.,* 1992). A third class of proteins in the HSP70 family is the glucose-regulated protein 78, abbreviated as GRP78 (Shiu *et al.,* 1977; Lee, 1987; Lui and Lee, 1991). GRP78 is not heat-inducible, however.

There is 75 to 100% amino acid sequence identity between HSC70 and HSP68 proteins within mammalian species and >50% homology between mammalian HSP70 molecules and those of *E. coli* and *Drosophila* (Hunt and Morimoto, 1985; Dworniczak and Mirault, 1987). A distinct characteristic of HSC70 and HSP68, as well as most other HSPs, is their function as chaperone molecules within cells. Such activity is conferred from the ability of HSPs to bind hydrophobic domains of proteins. Hydrophobic domains are not normally accessible on proteins with normal tertiary and quartenary structure but are readily accessible in proteins which have been denatured. HSC70 and its bacterial counterpart, DNAk, associate with denatured proteins at a higher affinity than for non-denatured proteins (Liberik *et al.,* 1991; Palleros *et al.,* 1991; Palleros *et al.,* 1992). With use of random oligomers, HSC70 has been reported to preferentially bind hydrophobic amino acids, particularly amino acids containing nonpolar side chains (Flynn *et al.,* 1991). This binding ability is localized within the final 150 amino acids of the C-terminal region of HSP70. This region contains repeated uncharged amino acids such as proline, glycine and alanine as well as amino acids with hydrophobic residues (Hunt and Morimoto, 1985; Sorger and Pelham, 1987). Hydrophobic domains for HSP70 are highly similar to

hydrophobic domains of other binding proteins, such as histocompatibility antigens, for example, which bind to and present protein to lymphocytes (Rippmann *et al.,* 1991).

Although association of HSC70 and HSP68 with proteins is not energy dependent, dissociation of HSP70 molecules from substrates requires hydrolysis of ATP (Flaherty *et al.,* 1991; McCarty and Walker, 1991). Partial proteolytic digestion studies on HSP70 molecules have determined that the ATPase activity resides within a 44 kDa fraction within the N-terminal portion of the protein (Chappell *et al.,* 1986; Milarski and Morimoto, 1989). This region displays sequence homology with ATP binding sites of protein kinases (Walker *et al.,* 1982; Hannink and Donoghue, 1985) and has structural similarities to hexokinase (Fletterick, 1975) and actin (Kabsch *et al.,* 1990; McKay, 1993). The ATPase activity of HSP70 may also be increased during heat shock, to promote association and prevent denaturation of cellular components. ATPase activity of DNAk, the bacterial counterpart of HSC70, increases 70-fold following heat shock (Liberek *et al.,* 1991; McCarty and Walker, 1991). It is not known, however, whether ATPase activity of mammalian HSP70 proteins increases during heat.

HSP70 proteins utilize protein binding for constitutive roles such as aiding in oligomeric disassembly and protein translocation within cells. HSC70 is an essential component of endocytosis by clathrin-coated pits. Clathrin is composed of three units, each containing a heavy chain and light chain (Ungewickell and Branton, 1981), which localize at the plasma membrane. Areas rich in clathrin are termed

coated pits and are responsible for the clustering and endocytosis of transmembrane receptors (Pearse, 1988). Following endocytosis, clathrin forms a polyhedral lattice around the endocytosis vesicle (Crowther and Pearse, 1981). HSC70 is responsible for clathrin lattice disassembly through processes dependent on ATP hydrolysis (Ungewickell, 1985; Chappell *et al.,* 1986). HSC70 disassembles clathrin oligomers by association with one of the two light chain regions of clathrin that contain a glycine and proline enriched region (DeLuca-Flaherty *et al.,* 1990). Following clathrin disassembly, vesicles proceed through endosomal pathways and the clathrin triskeleton is recycled (Rodman *et al.,* 1990).

HSC70 also associates with proteins during translation on free ribosomes. This association may aid in targeting proteins that are not folded correctly for degradation or to allow for proper refolding (Welch, 1992). HSC70 association also aids in translocation of proteins to the mitochondrium and endoplasmic reticulum (Pelham, 1989; Welch, 1992). Since protein unfolding is an essential prerequisite for translocation from the cytosol to the mitochondrium or endoplasmic reticulum (Schleyer and Neupert, 1985; Eilers and Schatz, 1986), HSC70 binding appears essential for translocation of proteins. The specificity of HSC70 for translocated proteins has not been determined but may involve N-terminal sequences of proteins. These sequences serve to target proteins for the mitochondrium or endoplasmic reticulum. Additionally, these signal sequences may promote HSC70 binding, since several signal sequences retard protein folding during translation (Park *et al.,* 1988). For mitochondrial proteins, HSC70 becomes dissociated as translocation progresses

to allow for proteins to proceed through the mitochondrial membranes (Deshaies *et al.,* 1988; Pfanner *et al.,* 1990). As proteins pass through the mitochondrial membranes, HSC70 localized within the mitochondrium binds proteins. Intramitochondrial association of HSC70 to proteins during translocation has been shown to be essential for successful translocation (Kang *et al.,* 1990) and probably aids to inhibit protein folding within the mitochondrium. Following translocation, HSC70 is dissociated from internalized proteins and proper folding of these proteins occurs with the aid of HSP60 (Ostermann *et al.,* 1989). Events involved with translocation of proteins to the endoplasmic reticulum appear to be similar to that of mitochondrial translocation events except that HSC70 and HSP60 are not involved with binding and folding of proteins once proteins enter the endoplasmic reticulum (Pelham, 1989; Welch, 1992).

HSC70 also has been implicated in localization of some proteins to the nucleus. Nuclear localization signals for proteins consists of a pair of identical and conserved seven basic amino acid residues, often separated by a ten to eleven amino acid spacer (Adam and Gerace, 1991; Dingwall and Laskey, 1991; Robbins *et al.,* 1991; Shi and Thomas, 1992). In some proteins, the spacer region contains proline residues which promote protein folding and thereby mask localization signals (Dingwall and Laskey, 1992). It has been postulated that HSC70 binds to the spacer region to inhibit folding, thus maintaining the access of nuclear localization sequences to nuclear receptors. Indeed, motifs less than 10 amino acids in length can be bound by HSC70 (Flynn *et al.,* 1989). Additionally, HSC70 and HSP68 contain

nucleus localization sequences (Milarski and Morimoto, 1989; Yamasaki *et al.,* 1989). These proteins do not localize to a great extent within the nucleus during non-stressed conditions, but nuclear localization has been observed following heat shock (Pelham, 1984; Velazquez and Lindquist, 1984; Welch and Ferisco, 1984; Welch and Mizzen, 1988).

Recent studies have determined that HSC70 is a component of progesterone receptor complexes in chickens and humans (Kost *et al.,* 1989; Onate *et al.,* 1991). HSC70 maintains association with receptors in the presence or absence of progesterone binding (Kost *et al.,* 1989; Onate *et al.,* 1991; Smith *et al.,* 1992). The importance of HSC70 binding to steroid receptors is unclear. Such association may be required for stabilizing progesterone receptor complexes to optimize binding of progesterone or to assist HSP90 molecules to prevent dimerization and/or DNA binding of non-steroid bound receptors. In contrast, Sanchez *et al.* (1990) has suggested that association of HSC70 with steroid receptors is not essential for performance of the complex since HSC70 does not associate with progesterone receptors in mice.

Expression of HSP68, but not HSC70, increases coincidentally with DNA synthesis (Milarski and Morimoto, 1986). Functions of HSP68 during this time, however, have not been determined. Perhaps HSP68 serves to stabilize proteins associated with DNA replication. Additionally, synthesis of HSP68 increases with cellular transformation or virus invasion (Pinhasi-Kimhi *et al.,* 1986) and HSP68 can be found associated with viral proteins (White *et al.,* 1988; Li *et al.,* 1990; Koskinen

et al., 1991), implying that HSP68 may be important during early viral invasion. Interestingly, increased cellular sensitivity to heat shock ensues in virus-infected cells (Li *et al.,* 1990). Perhaps thermosensitivity occurs because of the preferential binding of HSP68 to viral proteins rather than to other cellular components during heat shock.

HSP70 and heat shock. Heat shock induces a 10 to 20 fold increase in HSP68 synthesis and 2 to 3 fold increase in HSC70 synthesis (Milarski *et al.,* 1989). Increased expression of HSC70 and/or HSP68 during heat shock appears to be essential for conferring thermal resistance in many cell types. With induction of thermotolerance (*i.e.,* increased resistance to a severe heat shock after prior exposure to a mild heat shock), translational activity (Mizzen and Welch, 1988) and cell survival (Li and Werb, 1982; Li, 1985; Mivechi and Li, 1985; Widelitz *et al.,* 1987) are correlated positively with accumulation of HSP68. Additionally, HSP68 expression is more highly correlated with cellular tolerance to heat shock than other classes of HSPs in human fibroblasts (Mizzen and Welch, 1988) and several variants of cell lines (Li and Werb, 1982; Laszlo and Li, 1985; Li, 1985; Mizzen and Welch, 1988; Mivechi and Rossi, 1990). More direct evidence also implicates that HSP70 proteins are important in conferring cellular resistance to heat shock. In murine oocytes, which lack the ability to transcribe HSP68 and HSC70, microinjection of mRNA for HSC70 caused enhanced resistance to heat shock (Hendrey and Kola, 1991). Similar observations also have been observed following overexpression of HSC70 (via transfection) in CV1 monkey cells and mouse fibroblasts (Angelidis *et al.,* 1991; Li

et al., 1991). Injection of antibodies to HSP68 and HSC70 (Riabowol *et al.,* 1988) or injection of DNA sequences which compete for cis-acting elements that regulate HSP gene expression (Johnson and Kucey, 1988) decreased resistance of cells to heat shock. However, HSP68 may not be essential for all types of cells to confer thermal resistance or undergo thermotolerance. Hatayama *et al.* (1991) concluded that thermotolerance was induced in Chinese hamster V79 cells without increased synthesis of HSP68. Perhaps the essential role of HSP68 in conferring thermal resistance may be determined by differential cellular expression of additional HSPs or other biochemical mechanisms that may substitute for HSP68.

One mechanism by which HSP70 proteins induce cellular resistance to heat shock likely involves association with malformed cellular components. HSC70 and HSP68 have increased affinity for denatured proteins and this binding persists longer for denatured proteins than for non-denatured proteins (Liberik *et al.,* 1991; Palleros *et al.,* 1991; Palleros *et al.,* 1992). However, the importance of this activity in conferring thermal resistance is unknown, since the degree of cellular damage caused by the existence of malformed proteins or the degree of alleviation exerted by HSP68 following association with malformed proteins has not been determined.

HSP70 may aid in conferring cellular resistance to heat shock by preventing heat shock induced damage to ribosomal components. Although the rate of ribosomal RNA synthesis and ribosome assembly decreases during heat shock, increased denaturation and aggregation of ribosomal components occurs within the nucleolus (Welch and Suhan, 1986). Both HSP68 and HSC70 accumulate in the

nucleus after heat shock, particularly within the nucleolus (Pelham, 1984; Velazquez and Lindquist, 1984; Welch and Feramisco, 1984; Welch and Mizzen, 1988). The presence of HSP68 and HSC70 within the nucleolus could aid in stabilizing ribosomal components, possibly by binding to misfolded components, preventing aggregation and inactivating denatured components. Translational activity of HeLa cells can be increased during and after heat shock with prior exposure to a mild heat shock (Mizzen and Welch, 1988). Induction of thermotolerance increases the rate of HSP68 localization to the nucleolus and exit from the nucleolus after heat shock (Welch and Mizzen, 1988). Hence, induction of thermotolerance seemingly enhances HSP68 complex formation with nucleolar structures and may hasten their protection or repair during and after heat shock. The protective role of HSP68 for ribosomal components is also implied by findings that HSP68 accumulates in several additional regions which contain ribosomes, particularly in the perinuclear regions, plasma membrane and within dense structures (Welch and Suhan, 1986).

The ability of HSP68 to protect translational machinery may, however, depend on the stage of the cell cycle. As mentioned previously, synthesis of HSP68 is increased during DNA replication, although induction of thermotolerance cannot be achieved in HeLa cells during DNA synthesis (Milarski and Morimoto, 1986). HSP68 is not localized within the nucleolus during this time; instead the protein is apparently complexed with other cellular components (Milarski *et al.,* 1989). Translational machinery and possibly other cellular components that cannot complex with HSP68 may be more sensitive to heat shock during periods of DNA replication.

HSP90. In addition to being one of the most abundant proteins in mammalian cells under non-stressed conditions, expression of this phosphoprotein (83 to 94 kDa molecular weight; pI of 5.1 to 5.8) increases with heat shock (Welch *et al.,* 1983; Moore *et al.,* 1987; Iannotti *et al.,* 1988; Iwasaki *et al.,* 1989). Also included in this class of HSPs is GRP94, a protein localized in the endoplasmic reticulum whose synthesis is enhanced by glucose starvation but not by heat shock (Lee, 1987; Liu and Lee, 1991). As many as 12 isoforms for HSP90 can be observed by 2-dimensional electrophoresis. These represent differentially phosphorylated proteins from two genes that share >70% protein sequence homology (Welch *et al.,* 1983; Rebbe *et al.,* 1987; Iannotti *et al.,* 1988; Hickey *et al.,* 1989). HSP90 is distributed within the cytoplasm under non-stressed conditions and maintains primarily a cytoplasmic localization during heat shock, with some additional concentration of HSP90 around the cell membrane and within the nucleus (Kelley and Schesinger, 1982; Carbajal *et al.,* 1986; Collier and Schlesinger, 1986; Van Bergen en Henegouwen *et al.,* 1987). Although HSP90 binds to cellular proteins through hydrophobic interactions (Iwasaki *et al.,* 1989), HSP90 lacks intrinsic ATP hydrolysis activity (Hardesty and Kramer, 1989). With the use of coprecipitation studies, HSP90 has been observed to associate with actin (Koyasu *et al.,* 1986; Nishida *et al.,* 1986) and tubulin (Pratt *et al.,* 1989) in a calmodulin-dependent manner. Perhaps HSP90 binding to other cellular components is regulated similarly. Indeed the structural homolog, GRP94, exhibits calmodulin-dependent binding to proteins (Kang and Welch, 1991; Melnick *et al.,* 1992).

One constitutive role of HSP90 is complexing with steroid hormone receptors in the absence of steroid binding (Carson-Jurica *et al.,* 1990; Smith and Toft, 1993). Upon steroid binding, HSP90 is dissociated and receptor dimerization and binding of receptors to DNA occurs (Beato, 1989; Carson-Jurica *et al.,* 1990; Smith and Toft, 1993). HSP90 appears to be essential for regulating transcriptional activity of steroid receptors since disruption of the receptor/HSP90 complex results in receptor dimerization and DNA binding in the absence of steroid (Lindquist and Craig, 1988). Inhibition of intrinsic receptor activity through HSP90 binding likely occurs because the DNA binding site of receptors is masked, since HSP90 contains a negatively charged α-helical domain which may associate with the DNA binding domain (Bilinski *et al.,* 1988) or because HSP90 presents a stearic hindrance for receptor dimerization, which is a critical prerequisite for transcriptional activity (Pratt *et al.,* 1989; Demarco *et al.,* 1991).

An additional role for HSP90 within cells pertains to the possible regulation of tyrosine kinase specific enzymes. HSP90 complexes with the oncogenic transforming protein $pp60^{src}$, which is a tyrosine kinase oncogenic product of the Rous sarcoma virus (Sefton *et al.,* 1978; Brugge *et al.,* 1981; Oppermann *et al.,* 1981). When the kinase was complexed with HSP90 and an associated 50 kDa protein, it was inactive; however full activity of $pp60^{src}$ was achieved by dissociation of HSP90 and associated proteins. It has been speculated by Brugge (1986) that complex formation is essential for eliminating the tyrosine kinase activity of the oncogenic product until the kinase has localized to the plasma membrane, its site of activity.

Several additional retrovirus-encoded oncogenic products that contain tyrosine kinase activity likewise complex with HSP90 (Brugge, 1986), suggesting that HSP90 binding assists in the transport of oncogenic and possibly inherent cellular tyrosine kinases throughout the cell.

HSP90 is important for conferring thermal resistance; reducing levels of cellular HSP90 by antisense treatment resulted in reduced growth and survival of heat-shocked L-cells (Bansal *et al.,* 1991). Since HSP90 associates with cytoskeleton components (Nishida *et al.,* 1986; Koyasu *et al.,* 1989; Pratt *et al.,* 1989), it is possible that the protein serves to stabilize the cytoskeleton during heat shock. Indeed, severe heat shock promotes cytoskeleton disassembly, which can be inhibited with induction of thermotolerance (Welch and Mizzen, 1988; Shyy *et al.,* 1989). Alternatively, HSP90 may aid in conferring cellular resistance to heat shock by decreasing translation rate within heat shock cells. Such an action has been observed in reticulocytes, as association of HSP90 with eukaryotic initiation factor 2α kinase increases the enzymes ability to inhibit translation (Rose *et al.,* 1987).

HSP27. HSP27 is represented by proteins of 25 to 30 kDa with pI range of 5.9 to 6.3 which are usually encoded by a single gene in mammalian cells (Hickey *et al.,* 1986; Arrigo and Welch, 1987; Faucher *et al.,* 1993). Most proteins in the HSP27 family are characterized by low methionine content and high degree of phosphorylation (Kim *et al.,* 1983b; Arrigo and Welch, 1987). The level of serine and threonine phosphorylation for HSP27 is highly variable and may be dependent, in part, on serum factors, calcium ionophores or types of cellular stress (Kim *et al.,*

1983b; Arrigo and Welch, 1987). There is considerable sequence variation between HSP27 of different organisms, although hydrophilic and hydrophobic domains within the C-terminus are highly conserved and related to domains found on α-crystallin (Neumann *et al.,* 1989).

A possible function of HSP27 during non-stressed conditions may be to regulate RNA activity. HSP27 forms aggregates, like α-crystallin, which contain mRNA (approximately 400 kDa) and localize around the Golgi under non-stressed conditions (Collier and Schlesinger, 1986; Arrigo and Welch, 1987; Arrigo *et al.,* 1988). HSP27 may also be involved in developmental processes in certain organisms. In *Drosophila*, for example, expression of HSP27 is altered during development and differentiation (Cheney and Shearn, 1983; Pauli *et al.,* 1990).

Synthesis of HSP27 occurs at low levels under unstressed conditions and increases 10 to 20 fold upon exposure to heat shock (Kim *et al.,* 1983b; Welch, 1985). HSP27 is capable of conferring thermotolerance to cells; transfection of murine cells with the HSP27 gene to induce overexpression increased thermal resistance of cells (Landry *et al.,* 1989). However, functions of HSP27 during heat shock remain speculative. During heat shock, HSP27 localizes within the nucleus to form aggregates as large as 2000 kDa (Collier and Schlesinger, 1986; Arrigo *et al.,* 1988). The presence of this protein may aid in stabilizing or regulating activity of RNA transcripts, given that constitutive RNA binding to HSP27 aggregates has been observed (Nover *et al.,* 1989).

Other HSPs. A single 110 kDa protein with a pI of 5.5 has been identified as a heat-induced protein in mammalian cells that is also expressed constitutively at low levels (Subjeck *et al.,* 1982; Subjeck *et al.,* 1983; Shyy *et al.,* 1986). Under non-stressed conditions, HSP110 is found throughout the cell but localizes to the nucleolus during heat shock (Subjeck *et al.,* 1983; Shyy *et al.,* 1986). Depletion of yeast HSP110 abolishes the ability of cells to undergo induced thermotolerance (Sanchez and Lindquist, 1990), implying that at least in yeast, HSP110 is essential for conferring thermal resistance.

HSP60 is a heat-inducible protein that is also constitutively expressed in mammalian cells. This protein has an apparent molecular weight of 58 to 64 kDa, pI of 5.8 and is localized within the mitochondria during both non-stress and heat shock conditions (McMullin and Hallberg, 1988; Waldinger *et al.,* 1989). Monomers and oligomers of this protein function as chaperones within the mitochondria. In particular, HSP60 is involved in assembly of monomeric proteins following transport through the mitochondrial membranes and formation of polymeric complexes (Ostermann *et al.,* 1989). Potential functions of HSP60 during heat shock are not defined. One possibility is that HSP60 may limit or correct altered protein structure that may occur within the mitochondria during heat shock. The mitochondria may be particularly susceptible to heat shock because free radical production may increase within the mitochondria during heat shock.

Ubiquitin (HSP8.5) is a 8.5 kDa protein that covalently binds to proteins and targets them for degradation through nonlysosomal pathways. Ubiquitin mediated

protein degradation is likely responsible for selective intracellular protein degradation (Chin *et al.,* 1982; Hershko *et al.,* 1982; Bachmair *et al.,* 1986). Ubiquitin activating enzyme (E1) utilizes ATP to modify ubiquitin conformation and increase affinity for proteins (Ciechanover *et al.,* 1984; Finley *et al.,* 1984). Following activation, ubiquitin binds to lysine residues of proteins targeted for degradation (Hershko *et al.,* 1984). This binding is regulated by ubiquitin's association with ubiquitin carrier protein (E2), which aids in targeting proteins, and by ubiquitin protein ligase (E3), which initiates ligation of ubiquitin to the protein (Hershko *et al.,* 1986; Ciechanover and Schwartz, 1989). Following binding of a single ubiquitin, poly-ubiquitination of the protein occurs, which targets proteins for degradation by an ATP-dependent protease to yield free amino acids and reusable ubiquitin (Ciechanover and Schwartz, 1989; Ciechanover *et al.,* 1990).

Binding of ubiquitin is regulated by the N-terminal amino acid of targeted proteins. While absence of methionine at the N-terminus will usually confer ubiquitination (Ciechanover *et al.,* 1990), ubiquitination is more prevalent in proteins containing basic N-terminal residues (histidine, arginine or lysine) or hydrophobic N-terminal residues (leucine, tryptophan, phenylalanine or tyrosine; Reiss *et al.,* 1988). Since most proteins contain methionine as the N-terminal residue, ubiquitin mediated proteolysis is likely a regulatory mechanism involved with degrading proteins that have been translated incorrectly. Additionally, translation of proteins containing N-terminal sequences that promote ubiquitination may also represent a mechanism to promote short lived cellular effects of certain proteins.

Ubiquitination of proteins that are damaged during heat shock may be an important mechanism to increase sensitivity of cells to heat shock. Indeed, heat-induced synthesis of ubiquitin has been observed in a number of species (Bond and Schlesinger, 1985; Ovsenek and Heikkila, 1988; Forance *et al.,* 1989b). However, the importance of this system may be questioned since rate of protein degradation decreases during heat shock in HeLa cells (Carlson *et al.,* 1987), possibly as a consequence of decreased activity of proteolytic enzymes.

HSP gene regulation. Increased synthesis of HSPs by heat shock and a number of other cellular stresses is regulated primarily by increased rate of transcription. Expression of HSPs following heat stress is induced by interaction of a trans-activating factor, termed heat shock factor (HSF), with specific cis-acting elements termed heat shock elements (HSE). The HSE consists of at least two continuous inverted repeats of nGAAn nucleotide sequences arranged either head-to-head (nGAAnnTTCn) or tail-to-tail (nTTCnnGAAn) and usually located within the first 300 bp upstream of the transcription start site of all genes encoding HSPs presented in this section (Perisic *et al.,* 1989; Nover, 1991) and of other heat-induced genes such as heme oxygenase and interleukin-7 (Mitani *et al.,* 1991; Wathelet *et al.,* 1987; Lupton *et al.,*1990). Transcriptional activation studies have determined that one HSE is adequate for transcription, but two or more elements are needed for maximal transcription (Pelham, 1982; Dudler and Travers, 1984; Topol *et al.,* 1985; Kay *et al.,* 1986; Klemenz and Gehring, 1986; Amin *et al.,* 1987). As with other elements involved with transcription regulation, HSE binding affinity to HSF is

higher at the HSE most proximal to the transcription start site and initial binding to this site promotes cooperative binding of distal sites to HSF (Topol, 1985; Kay *et al.,* 1986; Amin *et al.,* 1987). However, the ability of HSE sequences to promote transcription decreases with increased distance from the transcriptional start site (Amin *et al.,* 1987).

Although the possibility remains that low level HSF interaction with HSE could cause some expression of HSPs in the absence of cellular stress, constitutive expression of HSPs is mainly conferred by additional cis-acting elements and trans-activating factors within the 5' promoter regions of genes encoding HSPs (Tanguay, 1988). For example, the 5' flanking region of the human HSP68 gene contains a serum response element which probably regulates growth factor or cell cycle dependent expression (Wu *et al.,* 1987).

The trans-activating protein factor, HSF, interacts with HSE motifs to promote gene expression. In unstressed cells, HSF is found throughout the cytoplasm and nucleus in a monomeric form with low binding affinity to DNA. In response to heat shock, HSF forms trimers, localizes within the nucleus, and binds to HSE with high affinity (Wu, 1984; Sorger and Nelson, 1989; Westwood *et al.,* 1991; Jurivich *et al.,* 1992). However, HSF binding to HSE alone does not induce transcription. In yeast, trimerization and DNA binding occurs innately, but transcription is not initiated (Gross *et al.,* 1990; Jakobsen and Pelham, 1991). This phenomenon was also observed following microinjection of yeast HSF into *Xenopus* oocytes (Clos *et al.,* 1990) or murine erythroleukemia cells (Hensold *et al.,* 1990). Although yeast derived

HSF bound DNA, HSP gene expression did not occur in *Xenopus* or mammalian cells. Phosphorylation of HSF, which may alter HSF conformation, is a possible mechanism by which HSF-induced transcription is activated. This has been proposed since initiation of transcription for HSPs in yeast and other mammalian cells is correlated with serine/threonine phosphorylation of HSF (Krishnan and Pueppke, 1987; Sorger, 1990).

The structure of HSF, derived from DNA sequence analysis, has provided much information pertaining to the nature of HSF trimerization, DNA binding and activation of transcription. Cloning of HSF genes has revealed that HSF is expressed by a single gene in yeast and *Drosophila* (Clos *et al.,* 1990; Jacobsen and Pelham, 1991), by two genes in humans and mice (Sarge *et al.,* 1991; Schuetz *et al.,* 1991) and by three genes in tomatoes (Scharf *et al.,* 1990). Two highly conserved regions persist for HSF molecules among organisms. The first is a 188 amino acid motif in the N-terminal region that is responsible for DNA binding. Although this region contains no known binding motifs used by eukaryotic cells (Wiederrecht *et al.,* 1988; Clos *et al.,* 1990), it is considerably homologous to bacterial sigma DNA binding factors (Clos *et al.,* 1990). Perhaps a novel eukaryotic motif is utilized by this trans-activating factor. The second conserved region, located in the C-terminal region, is responsible for HSF trimerization (Rabindran *et al.,* 1993). This region contains coiled-coil motifs that serve as a flexible hinge to promote binding of all three N-terminal regions to DNA regions (Sorger and Nelson, 1989; Clos *et al.,* 1990; Westwood *et al.,* 1991). Additionally, C-terminal region mutagenesis studies have

revealed that a region of HSF maintains transient activation since alteration of this region induces constitutive transcriptional activation (Nieto-Sotelo *et al.,* 1990; Sorger, 1990). However, the mechanisms by which this region regulates transient transcriptional activity is not known.

Distinct HSFs, produced from different genes, appear to possess divergent roles in the heat shock response. Through use of antibodies, one factor, HSF1, was determined to be involved in transcriptional activation in response to heat shock, oxidative stress, heavy metals and amino acid analogues (Sarge *et al.,* 1993) whereas the second factor, HSF2, was responsible for hemin-induced differentiation of erythroleukemia cells (Sistonen *et al.,* 1992). The third HSF found in tomatoes, HSF3, does not initiate transcription following any of the above stimuli (Morimoto, 1993). Therefore, specific signals, some of which may not be yet discovered, induce activation of transcription by specific isoforms of HSF.

Signalling systems for trimerization, DNA binding and transcriptional activation of genes encoding HSPs by HSF have not been completely determined. Much evidence lends support to a theory for HSF regulation by the constitutively expressed protein, HSC70 (Sorger, 1991; Lis and Wu, 1993; Morimoto, 1993). HSF binding to DNA can be induced in cytoplasmic extracts with heat shock, exposure to detergents or low pH (Larson *et al.,* 1988; Mosser *et al.,* 1990; Abravaya *et al.,* 1992; Mosser *et al.,* 1993) but addition of HSC70 blocks this effect (Abravaya *et al.,* 1992; Mosser *et al.,* 1993). This effect can be reversed with addition of ATP (Abravaya *et al.,* 1992), possibly due to ATP-induced dissociation of HSC70 from HSF. Similarly,

underexpression of HSC70 results in enhanced synthesis of other HSPs under non-stress conditions in *Drosophila* (Solomon *et al.,* 1991). It has been speculated (Sorger, 1991; Morimoto *et al.,* 1992; Morimoto, 1993) that HSC70, and possibly other constitutively produced HSPs, make up an abundant pool of excess HSPs which will associate with HSF under non-stressed conditions and inhibit HSF activation. During heat shock, HSC70 association with HSF becomes limiting since other cellular proteins that have been adversely affected by heat shock would compete with HSF for associating with HSC70. Consequently, unbound HSF would then be able to trimerize, become activated and initiate HSP synthesis. The expression of HSPs would then continue until an abundant pool of HSC70 and possibly other HSPs is re-established. However, researchers have been unable to determine whether an association between HSC70 and HSF exists in non-stressed cells. Therefore, it is also possible that other regulatory mechanisms are involved with signalling HSF activation.

An intriguing event that has prompted investigation is the speed at which HSPs are transcribed following initiation of heat shock; mRNA encoding HSPs have been observed to increase within minutes after heat shock (Wu, 1980; Sorger, 1991). In non-stressed *Drosophila* cells, regions of DNA encoding HSPs are maintained in a chromatin structure depleted of nucleosomes, where DNA unwinding has already occurred (Nacheva *et al.,* 1989). Transactivating factors bind to TATA boxes of these HSP genes during non-stressed conditions, promote RNA polymerase binding and synthesis of the first 17 to 37 base pairs of mRNA for HSPs (Rasmussen and Lis,

1993). The mechanism by which transcription is halted has not been elucidated. Upon HSF binding and activation, transcription resumes, likely as a result of DNA folding to make downstream DNA sequences accessible to polymerase. Alternatively, HSF binding may aid in recruiting additional RNA polymerases which take over for the stalled polymerase.

Translational processing is also involved in regulation of HSP synthesis. HSP transcripts are preferentially translated during periods of heat shock (Storti *et al.*, 1980; Scott and Pardue, 1981). However, mechanisms for this process have not been defined. In *Drosophila*, stability of mRNA for HSPs is increased during heat shock (DiDomenico *et al.*, 1982) and this may result in preferential translation during heat shock.

Glutathione

Antioxidants protect cells from secondary damage caused by free radicals generated by heat shock, other cellular insults and normal metabolism. Antioxidants can act in two ways; by reacting directly with free radicals through electron transfer to neutralize free radicals or by acting through enzymatic processes to remove free radicals or correct damage to cellular macromolecules caused by free radicals. The antioxidant, glutathione (GSH), is a prominent water-soluble intracellular antioxidant that acts through both of these processes. Glutathione is a tripeptide (γ-glutamyl-cysteine-glycine) present intracellularly at concentrations of 500 μM to 10 mM while being undetectable extracellularly, except in the liver (Kosower, 1976; Meister and Anderson, 1983). It is present in cytoplasm and mitochondria either in the reduced

form (GSH) or as a dimer formed from disulfide bond formation between two oxidized molecules (GSSG). An overall summary of GSH metabolism, transport, recycling and functions are presented in Figure 2-1.

GSH synthesis, transport and recycling. GSH synthesis is dependent on transport of precursor amino acids into cells. Cysteine, glycine and glutamine residues are transported into cells through amino acid transport systems (Griffith *et al.,* 1979; Meister and Anderson, 1983). Following transport, glutamine is metabolized to glutamic acid by a two-step enzymatic process (γ-glutamyl cycle) requiring hydrolysis of ATP (Meister and Anderson, 1983). Reduced GSH is synthesized by a two-step process which is positively regulated by the availability of cysteine (Meister and Anderson, 1983). Cysteine and glutamic acid react with γ-glutamylcysteine synthetase to yield γ-glutamylcysteine (Gipp *et al.,* 1992). This reaction is rate limiting in the GSH synthesis process, requires ATP hydrolysis and is feedback-inhibited by GSH (Richman and Meister, 1975; Wirth and Thorgeirsson, 1978). GSH is formed by coupling glycine to γ-glutamylcysteine in an ATP-requiring reaction catalyzed by GSH synthetase (Richman and Meister, 1975; Wirth and Thorgeirsson, 1978). GSH is maintained predominately in the reduced form (as much as 99%; Carlberg and Mannervik, 1977; Griffin and Meister, 1979a; Griffith and Meister, 1979b) by the actions of GSH reductase, which uses NADPH derived primarily from oxidative processes of glycolysis to reduce GSSG (Thieme *et al.,* 1981; Williams *et al.,* 1982; Chung *et al.,* 1991).

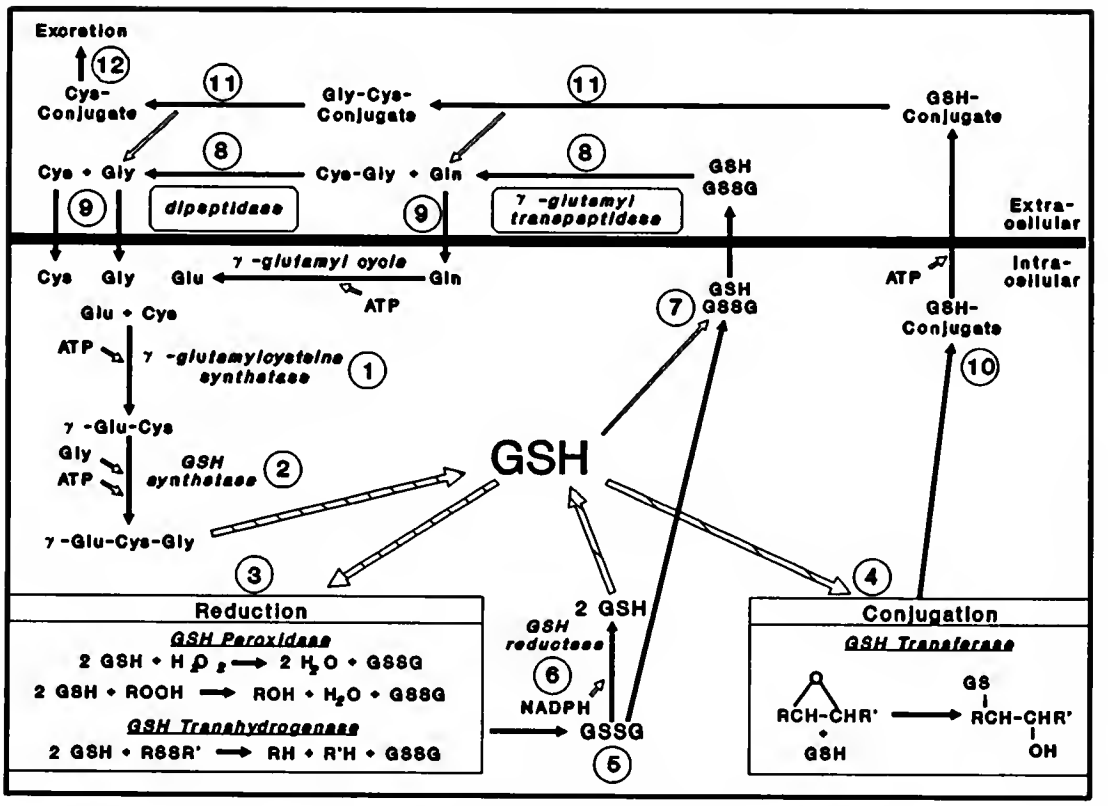

Figure 2-1. Synthesis, utilization and recycling of GSH within cells. A two step process involving γ-glutamylcysteine synthetase (1) and GSH synthetase (2) is responsible for synthesis of GSH within cells. The major function of intracellular GSH is to reduce free radicals or other electrophilic compounds (3) or to conjugate electrophilic compounds (4). These processes occur through direct reaction with GSH and via enzymatic-driven reactions. Reduction events produce the oxidized form, GSSG (5), which is readily converted to a reduced state by GSH reductase (6). Some GSH and GSSG is lost from the cell by passive diffusion (7) across the cell membrane and degraded on the extracellular surface by membrane-bound enzymes (8). Constitutive amino acids are then transported back into the cell for synthesis of GSH (9). Conjugates of GSH are transported out of the cell (10). Subsequently, glutamine and glycine residues are released by cleavage (11) and transported into the cell (9) whereas the remaining cysteine conjugate is targeted for excretion in urine (12).

Loss of intracellular GSH occurs as a result of transport of GSH, GSSG and GSH conjugates out of the cell through undefined systems. Loss of GSSG exceeds that of GSH because of its increased cell membrane permeability (Griffith and Meister, 1979a; Griffith and Meister, 1979b; Rouzer *et al.,* 1982). Following transport, GSH and GSSG are readily metabolized by γ-glutamyl transpeptidase and dipeptidase enzymes; these are soluble, membrane-bound proteins localized on the extracellular surface of cells (Griffith *et al.,* 1978; Hughey *et al.,* 1978; Meister, 1981; Okajima *et al.,* 1981). Glycine, glutamine and cysteine can then be utilized for resynthesis of GSH upon transport back into the cell.

Direct reactions of GSH. The ability of GSH to donate hydrogen for reduction of free radicals or other electrophilic compounds can occur without the aid of enzymes (Figure 2-1; Kosower, 1976). One reaction involves conversion of H_2O_2 and other peroxides (R_2O_2) to H_2O or ROH and GSSG (Mills, 1960; Pirie, 1965). The major source of H_2O_2 in cells is as a result of dismutation of $\cdot O_2^-$ by superoxide dismutase (Chance *et al.,* 1979). Since $\cdot O_2^-$ is produced as a result of electron transfer, superoxide dismutase is localized within the mitochondrial membrane as well as in the endoplasmic reticulum and peroxisome (Chance *et al.,* 1979). Lipid peroxides are formed from reaction of $\cdot OH$ with unsaturated fatty acids in membranes. Secondly, GSH reacts directly with disulfide bonds of other compounds to yield reduced sulfhydryl residues and GSSG (Schöberl and Gräfje, 1958; Thelander and Reichard, 1979; Mannervik and Axelsson, 1980). A third reaction of GSH involves direct conjugation of the cysteine residue of GSH with residues of

partially oxidized molecules such as heavy metals (Dierickx, 1982), ethanol (Hetu *et al.,* 1982), steroid derivatives (Benson *et al.,* 1977) and exogenous drugs (Chasseaud, 1979). Following GSH conjugation, transport out of the cell occurs by an ATP-dependent transport system (Ishikawa, 1989; Kobayashi *et al.,* 1990; Ishikawa, 1992). Within the extracellular milieu, γ-glutamyl and glycine residues of GSH are rapidly removed from the conjugate by γ-glutamyl transpeptidase and dipeptidase, respectively (Griffith *et al.,* 1978; Hughey *et al.,* 1978; Meister, 1981; Okajima *et al.,* 1981). Metabolism of the conjugate results in the formation of mercapturic acids, which are targeted for excretion in urine.

In addition to its intracellular functions, GSH may also directly reduce or conjugate molecules extracellularly. This activity is probably limited to the liver, because GSH concentrations range from 1 to 20 μM in liver blood plasma and 1 to 6 mM within bile, whereas extracellular concentrations of GSH are undetectable in other extracellular locations (Anderson *et al.,* 1980; Meister, 1984). The proposed role for extracellular GSH in the liver is reduction and conjugation reactions with toxic electrophilic compounds (Meister and Anderson, 1983; Avissar *et al.,* 1989). Increased extracellular GSH concentrations in liver result from low activity of γ-glutamyl transpeptidase and dipeptidase (Griffith and Meister, 1979a; Griffith and Meister, 1979b). In contrast to the liver, the activity of these catabolic enzymes is high in extracellular compartments of other tissues, particularly those which contain high secretory or absorptive functions (Griffith and Meister, 1979a; Griffith and

Meister, 1979b). High levels of γ-glutamyl transpeptidase and dipeptidase probably ensure that GSH and GSSG concentrations remain low extracellularly.

Enzyme-mediated reactions of GSH. Reduction of H_2O_2 and other peroxides by GSH is catalyzed by GSH peroxidases. Several isoforms of GSH peroxidase have been characterized, most of which require selenium as an electron donor (Forstrom *et al.,* 1978; Zakowski *et al.,* 1978). However, selenium-independent peroxidases have also been identified which, while not reacting with H_2O_2, can reduce other peroxides, particularly from the lipoygenase pathway of arachidonic acid metabolism (Lawrence and Burk, 1976; Burk *et al.,* 1978). Also, GSH peroxidase-mediated reactions have also been implicated in reducing molecules and other antioxidants that have reacted with free radicals (Wayner *et al.,* 1985; Frei *et al.,* 1988).

Thiol-disulfide bond exchanges of proteins with GSH is regulated by GSH transhydrogenases. These enzymes, of which at least two classes exist, ensure that the intracellular GSH pool acts as a thiol redox buffer within cells (Chance *et al.,* 1979; Anderson and Meister, 1980; Meister and Anderson, 1983). Protein disulfide isomerase is a GSH-dependent enzyme localized within the endoplasmic reticulum which serves to reduce disulfide bonds of proteins, reversing protein thiol oxidation and aiding in regulating tertiary protein structure (Mannervik and Axelsson, 1980). A second class of transhydrogenase enzymes, ribonucleotide reductase enzymes, aid in conversion of RNA to DNA. The proteins thioredoxin and glutaredoxin, rather than GSH, are the preferred electron donors for this enzyme. In turn, GSH aids in

reduction of oxidized thioredoxin and glutaredoxin (Thelander and Reichard, 1979; Hoog *et al.,* 1982).

Conjugation of sulfhydryl linkages between GSH and other molecules can be catalyzed by GSH S-transferases both intracellularly and extracellularly (Boyland and Chasseaud, 1969; Avissar *et al.,* 1989). Conjugation of GSH, as mediated by GSH S-transferase, also plays a role in physiological responses to stress. For example, leukotriene C_4 is formed from conjugation of GSH to leukotriene A_4 and is a local mediator of inflammation within tissues (Rouzer *et al.,* 1982; Bach *et al.,* 1984). Following export of leukotriene C_4 from the cell, removal of γ-glutamyl and glycine residues from GSH results in the formation of leukotriene D_4 and E_4, respectively.

Importance of GSH in stressed cells. The essential role of GSH in maintenance of cell function can be observed in several tissues. Decreased intracellular concentrations of GSH causes damage to skeletal muscle (Mårtensson and Meister, 1989), lung (Mårtensson *et al.,* 1989) and intestinal mucosa (Mårtensson *et al.,* 1990) and has been associated with numerous diseases (Uhlig and Wendel, 1992). The most prominent effect of decreased GSH concentrations is kidney damage. The kidney is probably more sensitive to decreased GSH concentrations because of its high rate of GSH metabolism and synthesis. Nephrotoxicity can be induced by treatment with D,L-buthionine-S,R-sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase (Griffith *et al.,* 1979; Griffith and Meister, 1979c), or by administration of cisplatin, a widely used anticancer agent (Harder and Rosenberg, 1970; Gottlieb and Drewinko, 1975) which is a nonspecific inhibitor of enzymes

requiring thiol cofactors (Aull *et al.,* 1979; Smith and Douglas, 1989). Detrimental effects of BSO and cisplatin can be reversed by oral or intraperitoneal administration of GSH (Aw *et al.,* 1991; Bump *et al.,* 1992). Exogenous administration of GSH probably affects intracellular GSH only after being metabolized extracellularly and resynthesized intracellularly after transport of amino acids into the cell. A membrane-permeable form of GSH, monoethyl GSH ester, is a more effective form of GSH for increasing delivery to cells because it is readily transported into the cell (Anderson and Meister, 1989). Although intraperitoneal injection of both GSH and monoethyl GSH ester reduced cisplatin-induced nephrotoxicity in mice, monoethyl GSH ester was effective at lower doses than GSH (Anderson *et al.,* 1990).

Intracellular GSH has been proposed to increase thermal resistance of cells. Variants of Chinese hamster fibroblasts containing higher concentrations of GSH at normal temperatures were more resistant to heat shock compared with variants containing less GSH (Shrieve *et al.,* 1986). This effect was diminished by depletion of GSH with BSO (Shrieve *et al.,* 1986). During heat shock, GSH concentrations are greatly increased within the mitochondrion (Freeman and Meredith, 1988), a primary site of free radical production.

Additionally, increased synthesis of GSH occurs in association with induction of thermotolerance. Intracellular GSH concentrations increased 2 to 3 fold following exposure to an initial heat shock that made V79 cells, murine mammary adenocarcinoma cells and postimplantation rat embryos resistant to a subsequent severe heat shock (Mitchell *et al.,* 1983; Russo *et al.,* 1984; Jones and Douple, 1990;

Harris *et al.,* 1991). Induction of thermotolerance by administration of ethanol, instead of a mild heat shock, likewise increased intracellular GSH concentrations (Mitchell *et al.,* 1983). Moreover, induction of thermotolerance was inhibited by BSO (Mitchell *et al.,* 1983; Russo *et al.,* 1984; Jones and Douple, 1990; Harris *et al.,* 1991) and Chinese hamster ovary cells gained resistance to heat shock if microinjected with GSSG (Lumpkin *et al.,* 1988) or GSH (Kapiszewska and Hopwood, 1988) prior to heat shock. Similar observations have also been made in mouse embryos. Aréchiga *et al.* (1992) reported that induction of thermotolerance in murine morulae was abolished with administration of BSO and whereas thermotolerance was increased by increasing intracellular GSH concentrations. The mechanism for induction of GSH synthesis during heat shock has not been defined but possibly is caused by increased activity of enzymes involved in GSH synthesis or by decreased GSH transport from the cell.

Enzymes which utilize GSH for removal of free radicals are also affected by heat shock. In a thermally resistant mouse embryonic cell line, activity and synthesis of superoxide dismutase and GSH peroxidase were increased 2 to 3 fold by heat shock (Omar *et al.,* 1987). GSH may also regulate HSP synthesis, although this has not been established with certainty. Russo *et al.* (1984) found that GSH depletion in V79 cells diminished HSP68 synthesis in response to heat shock. In contrast, heat-induced synthesis of HSP68 and other HSPs was not affected by GSH depletion in rat postimplantation embryos (Harris *et al.,* 1991).

Taurine

Taurine ($^{+}NH_3$-CH_2-CH_2-SO_3^{-}; 2-aminoethanesulfonic acid) is a ß-amino acid synthesized by metabolism of cysteine. Taurine is a weak antioxidant which reacts with electrophilic compounds through reactions involving its sulfonic acid (Aruoma *et al.,* 1988). While only a weak antioxidant, concentrations of taurine are very high in specific cells and tissues, and at these sites an antioxidant role for taurine or its precursor molecule, hypotaurine may be important. Taurine and hypotaurine may play a role during embryonic development since millimolar concentrations of these amino acids are present in uterine fluids and oviductal cells (Fahning *et al.,* 1967; Van der Horst and Brand, 1969; Casslén, 1987). Taurine is a non-essential amino acid in most species; cats appear to be the exception because of low activity of enzymes required for taurine synthesis (Hardison *et al.,* 1977).

Taurine synthesis, transport and localization. Two primary routes of taurine biosynthesis exist: 1) sequential oxidation of cysteine to produce 3-sulfinoalanine followed by cysteic acid and subsequent decarboxylation to form taurine, or 2) decarboxylation of 3-sulfinoalanine to produce hypotaurine followed by oxidation to produce taurine (Wright *et al.,* 1986). Taurine is present in serum at concentrations of 100 μM but can be transported into cells to produce intracellular concentrations of up to 50 mM (Pasantes-Morales *et al.,* 1972; Cohen *et al.,* 1973). Such transport is achieved through a sodium- and energy-dependent transport system with a apparent Km of 15 to 20 μM (Schmidt, 1980; Tallan *et al.,* 1983). This transport

system can be inhibited by precursors of taurine, particularly by hypotaurine and alanine (Tallan *et al.,* 1983).

Intracellular concentrations of taurine are usually high in cells which produce large quantities of free radicals, suggesting taurine serves as an antioxidant in these cells. For example, taurine is present in high concentrations within the retina (Pasantes-Morales *et al.,* 1972; Cohen *et al.,* 1973), where it protects photoreceptors from free radical- or illumination-induced damage (Pasantes-Morales *et al.,* 1984). Likewise, taurine represents as much as 60% of the free amino acids within phagocytotic cells (Fukuda *et al.,* 1982). Taurine has also been shown to protect skeletal and cardiac muscle from exposure to external agents (Huxtable and Lippincott, 1981; Kramer *et al.,* 1981), protect cell membranes from free radical damage (Nakamori *et al.,* 1990), and protect bovine lymphocytes from damage caused by heat shock (Malayer *et al.,* 1992).

Free radical-scavenging properties of taurine. The antioxidant capabilities of taurine do not appear to involve highly specific scavenging of $\cdot O_2^-$, H_2O_2 or $\cdot OH$ radicals. Aruoma *et al.* (1988) demonstrated that taurine does not readily react with these free radicals, probably because the sulfonic acid group of taurine is not an optimal substrate for such free radicals. In contrast, because of its highly reactive sulfinic acid group, hypotaurine is highly reactive with all of these free radicals (Aruoma *et al.,* 1988; Green *et al.,* 1991). Taurine can readily scavenge the free radical hypochlorous acid (HOCl; Thomas *et al.,* 1986). This reactive molecule, particularly prevalent in neutrophils, is produced by the oxidation of chloride ions

(Harrison and Schultz, 1976; Morrison and Schonbaum, 1976) and causes formation of toxic aldehyde derivatives upon reaction with cellular components (Wright *et al.,* 1986). In contrast, chlorination of taurine by HOCl yields a stable chloramine with a half-life of ~2.5 d (Weiss *et al.,* 1982; Thomas *et al.,* 1983). Although taurine-chloramine still contains reactive properties, its degree of reactivity is greatly reduced compared with HOCl (Wright *et al.,* 1986).

Additional properties of taurine. Taurine is a prominent conjugate of bile salts. Its proposed role in the bile is conjugation and detoxification of potentially damaging components in biliary fluid, rather than affecting the absorption of lipids (Roy *et al.,* 1982; Dorvil and Yousef, 1983; Emudianughe *et al.,* 1983). Taurine may regulate osmotic pressure in cells, particularly in the eye, where taurine administration has been observed to inhibit swelling (Pasantes-Morales and Cruz, 1983). This amino acid may also be a neurotransmitter molecule, since taurine is released after photoexcitation (Pasantes-Morales and Quesada-Carabez, 1981). Calcium homeostasis may be regulated by taurine in some cells. In retina, taurine stimulates calcium influx in the presence of low intracellular calcium concentrations, whereas at high calcium concentrations, uptake is inhibited by taurine (Lopez-Colome and Pasanto-Morales, 1981).

Taurine is also an important amino acid during development. Taurine is present in high concentrations in milk, including colostrum in dairy cows (Sturman, 1986). In cats and rats, taurine deficiency increased the incidence of stillborn offspring, decreased birth weight, impaired post-natal growth and increased the

incidence of photoreceptor degeneration (DeLa Rosa and Stipanuk, 1984; Bonhaus *et al.,* 1985; Jacobsen *et al.,* 1987; Rapp *et al.,* 1988).

Taurine and hypotaurine also enhance spermatozoa viability. These amino acids are present in millimolar concentrations in semen from bulls, boars, guinea pigs, and humans (Van der Horst and Grooten, 1966; Johnson *et al.,* 1972; Meitzel *et al.,* 1980; Holmes *et al.,* 1992). Administration of hypotaurine increased motility of sperm (Leibfried and Bavister, 1982; Boatman *et al.,* 1990) and administration of hypotaurine or taurine stimulated the acrosome reaction (Mrsny *et al.,* 1979). Taurine and hypotaurine probably also serve antioxidant roles in semen since administration of hypotaurine and taurine inhibited lipid peroxidation of spermatozoa (Alvarez and Storey, 1983).

Taurine is present in uterine fluid at concentrations of 4 to 11 mM in humans (Casslén, 1987) and 1 to 2 mM in cattle (Fahning *et al.,* 1967). Although taurine concentrations have not been defined in oviductal tissue, tissue concentrations of hypotaurine are present in millimolar concentrations in ewe oviducts during the first few d following estrus (Van der Horst and Brand, 1969). Perhaps, as in semen, oviductal and uterine taurine and hypotaurine serves as important antioxidant molecules for the protection of spermatozoa, oocytes or embryos.

Thermoprotective property of taurine. It is possible that taurine can scavenge free radicals produced during heat stress. Malayer *et al.* (1992) found that taurine and alanine protected cultured bovine lymphocytes and murine embryos from heat

shock. Additionally, alanine reduced cytotoxic effects of heat shock on Chinese hamster ovary cells (Vidair and Dewey, 1987).

Other Antioxidant Molecules

In addition to GSH and taurine, Vitamin A, C and E aid in scavenging free radicals or electrophilic compounds from cells. In contrast to GSH and taurine, however, these antioxidants have not been examined for ability to protect cells from heat shock effects. Hence, this section will review antioxidant functions of vitamin A, C and E under situations other than heat stress.

Carotenoids (vitamin A). Carotenoids are a class of lipid-soluble molecules, some of which are precursors of vitamin A, which must be provided by the diet in most animals. β-carotene, retinol and other carotenoids are present throughout the body at tissue and plasma concentrations of 100 to 600 nM (Lehman *et al.,* 1988; DiMascio *et al.,* 1989). Carotenoids readily react with free radicals produced either by enzymatic or photochemical processes to yield formation of stable carbon radicals on methyl groups (Sies *et al.,* 1992). Carotenoids which react with $\cdot O_2^-$ radicals include ß-carotene, retinol, lycopene (an abundant-open-chained analogue), and the bile pigments bilirubin and biliverdin (Stocker *et al.,* 1987; DiMascio *et al.,* 1989; Conn *et al.,* 1991). The lipid solubility of this class of antioxidant suggests that they are able to protect membranes from lipid peroxidation. β-carotene and other carotenoids reduced peroxidation of lipids *in vitro* (Terao, 1989) and are more reactive with $\cdot O_2^-$ radicals than tocopherols (McDonagh, 1972).

Deficiency of β-carotene or retinol in dairy cattle has been associated with decreased pregnancy rates (Kuhlman and Gallup, 1942), increased incidence of spontaneous abortions and dead calves (Ronning *et al.,* 1959), and decreased growth rate (Ganguly *et al.,* 1980). Additionally, rate of spermatogenesis was reduced for bulls deficient in vitamin A (Hodgson *et al.,* 1946; Erbman *et al.,* 1984). Although mechanisms by which carotenoids aid in reproductive performance and growth rate are not defined, such consequences are probably caused at least in part by decreased antioxidant status.

Vitamin A has likewise been associated with fertility in pigs; administration of vitamin A or β-carotene around the time of breeding and early pregnancy decreased embryonic mortality (Brief and Chew, 1985; Coffey and Britt, 1989). Such beneficial effects are probably caused by beneficial effects of retinol and retinoic acid during embryonic development. Uterine concentrations of retinol and its associated binding protein (retinol-binding proteins) increase 7 to 8 fold during the second week of gestation in pigs (Trout *et al.,* 1992). Perhaps retinol and retinoic acid serve to limit detrimental effects of electrophilic compounds on embryonic development during this period. Vitamin A may also influence embryo development through additional mechanisms, such as promoting cell differentiation and proliferation (Schindler, 1986), regulation of gene expression (Chiocca *et al.,* 1988; Bedo *et al.,* 1989) and regulation of steroid synthesis (Talavera and Chew, 1988). It is also possible that cows require retinol during early pregnancy since retinol-binding proteins are secreted from bovine endometrium in cattle (Thomas *et al.,* 1992).

Ascorbic acid (vitamin C). Ascorbic acid, or vitamin C, is a water-soluble molecule which is an important antioxidant of extracellular fluids, being present in serum at concentrations of 30 to 150 μM (DiMascio *et al.,* 1989). Ascorbic acid is an efficient scavenger of $\cdot O_2^-$, H_2O_2, $\cdot OH$, HOCl and lipid peroxides with reaction rates similar to carotenoids and higher than tocopherols (Nishikimi, 1975; Halliwell *et al.,* 1987; Frei *et al.,* 1989; Sies *et al.,* 1992). Ascorbic acid probably serves as an important antioxidant molecule for spermatozoa. Ascorbic acid is present in seminal fluid at concentrations of 200 to 400 μM and has been shown to block oxidative damage of spermatogonal DNA (Fraga *et al.,* 1991).

Ascorbic acid also probably acts as an intracellular antioxidant because intracellular concentrations are similar to those in serum (DiMascio *et al.,* 1989). Ascorbic acid enhances the antioxidant status of membranes, in conjunction with GSH, by salvaging oxidized tocopherols and other electrophilic compounds (Packer *et al.,* 1979). Ascorbic acid is oxidized to dehydroascorbic acid upon reaction with tocopheroxyl radicals or other electrophilic compounds. This compound is then readily reduced to its reactive state by GSH transhydrogenase enzymes (Meister, 1992).

Reports of ascorbic acid deficiency related to reproductive performance in dairy cattle are lacking since cattle do not require dietary supplementation of vitamin C. However, ascorbic acid has been observed to promote fertility in cattle with reproductive problems. Ascorbic acid administration improved pregnancy rates in repeat breeder cows (Phillips *et al.,* 1941) and fertilizing ability of spermatozoa from

bulls with low fertility (Phillips *et al.,* 1940). Moreover, ascorbic acid concentrations in semen have been correlated positively with viability of bull spermatozoa (Phillips *et al.,* 1940).

Tocopherols (vitamin E). Tocopherols, or vitamin E molecules, encompass a group of lipid-soluble molecules that are considered to be the major antioxidant for protection of membranes from lipid peroxidation (Burton and Ingold, 1984; Horwitt, 1986). Although reactivity of tocopherols with free radicals is lower than for carotenoids and ascorbic acid (McDonagh, 1972; Sies *et al.,* 1992), tocopherols are present in tissues and serum at higher concentrations (2 to 40 μM; Lehman *et al.,* 1988; DiMascio *et al.,* 1989). Similarly to carotenoids, methyl groups on aromatic rings of tocopherols are responsible for reactions with $\cdot O_2^-$, H_2O_2, $\cdot OH$ and heavy metals (Pascoe and Reed, 1987). Oxidation of tocopherols produces formation of a stable tocopheroxyl radical, which can be reduced by reaction with ascorbic acid (Meister, 1992).

The role of vitamin E in reproduction for cattle has not been clearly established. Several studies suggest that vitamin E deficiency does not affect reproductive performance in cattle (Guillickson *et al.,* 1949; Schingoethe *et al.,* 1978). Prepartum administration of vitamin E and selenium improved fertility of cows in some studies (Segerson *et al.,* 1977; Shubin, 1988; Aréchiga *et al.,* 1994b) but not in other studies (Schingoethe *et al.,* 1982; Segerson and Libby, 1982; Kappel *et al.,* 1984). Fertility of spermatozoa was not affected by vitamin E administration to bulls (Salisbury, 1944). Several studies have determined that vitamin E and selenium

decreased the incidence of retained placentae (Segerson *et al.,* 1981; Harrison *et al.,* 1984; Eger *et al.,* 1985), metritis (Harrison *et al.,* 1984) and cystic ovaries (Harrison *et al.,* 1986). Beneficial effects of vitamin E and/or selenium during the periparturient period is probably caused by increased uterine contractility (Segerson and Libby, 1982) or by enhanced uterine neutrophil activity (Grasso *et al.,* 1990; Gilbert *et al.,* 1993).

Presence of Protective Mechanisms in Mammalian Embryos

Maternal heat stress may alter embryonic development and viability either directly or indirectly. Indirect effects of maternal heat stress, such as effects of heat stress on hormonal patterns and uterine secretions, have been described earlier in this review. Elevated uterine temperatures are likely to also directly affect embryonic development through alterations in lipid, protein and nucleic acid structure. The relative importance of embryonic versus reproductive tract effects during heat stress were assessed by Alliston and Ulberg (1961). In that experiment, pregnancy rates were reduced by transfer of embryos from heat-stressed ewes to nonstressed recipients, suggesting that heat stress compromised embryonic survival during early development. Additionally, transfer of embryos from nonstressed ewes into heat-stressed recipients reduced pregnancy rates to a lesser extent; suggesting that heat stress, in addition to directly influencing embryonic survival, exerts adverse effects on the uterus as well. This section will review available knowledge regarding direct effects of heat shock on embryonic function and discuss the mechanisms that embryos possess to limit deleterious effects of elevated temperatures.

Effects of elevated temperature on cultured embryos. Cultured mammalian embryos have been observed to be adversely affected by exposure to elevated temperature. Alliston *et al.* (1965) determined that exposure of 1-cell rabbit embryos to a heat shock of 40 C for 6 h decreased postimplantation development when embryos were subsequently transferred to recipients. Similarly, Gwasdauskas *et al.* (1992) determined that exposure of 1-cell mouse embryos to 39 C decreased subsequent *in vitro* development. Viability and development of murine morulae were decreased following exposure to 42 C or 43 C heat shock for 2 h (Aréchiga *et al.,* 1992; Malayer *et al.,* 1992).

Adverse effects of heat shock on embryos in culture are probably caused by direct effects of elevated temperatures on cellular components. Instability of lipids (Overath *et al.,* 1970; McElhaney, 1974; Yatvin, 1977; Bowler, 1981), denaturation of proteins (Privalov, 1979; Lepock *et al.,* 1983) and altered activity of enzymes (Ashburner and Bonner, 1979; Penafiel *et al.,* 1988; Walton *et al.,* 1989) occurs at temperatures of 40 to 45 C. These effects would adversely affect ion transport, signal transduction, metabolism of energy substrates, growth factor regulation and cytoskeleton integrity within embryos. Adverse effects of heat shock on cultured embryos may also include effects caused by free radicals. Several reports have observed that free radicals limit embryonic development under thermoneutral conditions. For murine embryos, free radical production can be observed during *in vitro* development (Nasr-Esfahani *et al.,* 1990; Goto *et al.,* 1992). In some mouse strains, embryonic development is "blocked" at the 2-cell stage *in vitro* (Goddard and

Pratt, 1983; Noda *et al.,* 1991). This block in development can be prevented by administration of antioxidants such as GSH (Legge and Sellens, 1991) and thioredoxin (Goto *et al.,* 1992), by administration of heavy metal chelators (Nasr-Esfahani and Johnson, 1992) or by administration of scavenging enzymes such as superoxide dismutase (Noda *et al.,* 1991; Goto *et al.,* 1992; Umaoka *et al.,* 1992). In addition, administration of taurine to mouse embryos from strains which do not undergo a developmental block promoted development to blastocyst stages in culture (Dumoulin *et al.,* 1992). Likewise, administration of hypotaurine in cultured hamster embryos improved survival of embryos following transfer to recipients (Barnett and Bavister, 1992).

Ontogeny of thermotolerance in embryos. Embryos gain resistance to heat shock as they progress in development. Alliston *et al.* (1965) determined that exposure to 40 C for 6 h decreased subsequent development of 1-cell rabbit embryos but had no effect on 2-cell embryos. Similarly, development of 1-cell mouse embryos exposed to 39 C for 96 h was decreased compared to controls maintained at 37 C, whereas 2-cell embryos were not affected by this heat shock (Gwasdauskas *et al.,* 1992). Similar observations have been made *in vivo*; embryonic development was decreased to a greater extent by maternal heat stress on d 1 of pregnancy than with heat stress on d 3 or later in sheep (Dutt, 1963). Similarly, maternal heat stress on d 1 of pregnancy decreased embryonic viability to a larger degree than with heat stress on d 20 of pregnancy in pigs (Tompkins *et al.,* 1967). An increased understanding of the role of HSPs and other systems in limiting effects of heat shock

in cells has provided some insight to the underlying cause for acquisition of thermotolerance during development.

HSP synthesis in embryos. An outline for the current knowledge of the ontogeny of induced thermotolerance, ontogeny of HSP synthesis and efficacy of antioxidants as thermoprotectants in murine embryos is presented in Figure 2-2. As described previously, the presence of thermoprotective mechanisms in cells can be determined by evaluating ability of cells to undergo induced thermotolerance. Muller *et al.* (1985) reported that exposure to a mild heat shock made mouse blastocysts more resistant to a subsequent, severe heat shock. There was no beneficial effect of exposure to a mild heat shock on 1-cell embryos, however. Further characterization of the ontogeny of induced thermotolerance has not been completed for mouse embryos. It is also not known whether induced thermotolerance occurs for embryos of other species.

Induced thermotolerance in cells is associated with increased production of HSPs; this may be true for embryos as well. Constitutive synthesis of HSC70 occurs in murine oocytes until the onset of oocyte maturation, when mRNA and protein levels decrease (Curci *et al.,* 1987; Curci *et al.,* 1991; Manejwala *et al.,* 1991). HSC70 mRNA and protein levels remain low until onset of transcription of the embryonic genome at the 2-cell stage. Constitutive synthesis of HSC70 and HSP68 resumes at the 2-cell stage in murine embryos but heat-induced synthesis of HSP70 has not been observed at the 2- or 8-cell stage although blastocysts undergo increased HSP70 synthesis in response to heat (Bensaude *et al.,* 1983; Wittig *et al.,* 1983; Morange

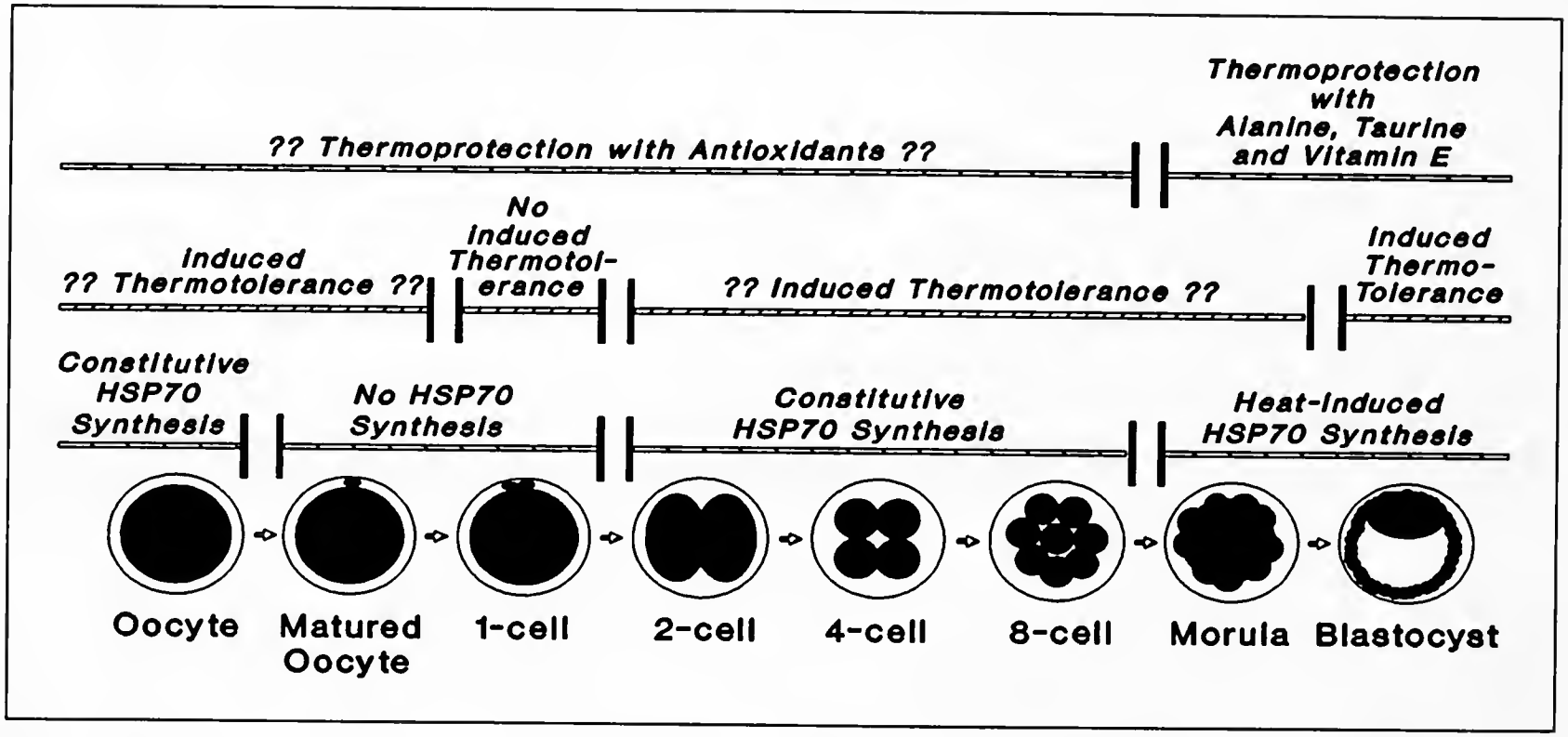

Figure 2-2. Events associated with the ontogeny of mechanisms conferring thermotolerance in murine embryos. Production of HSC70 mRNA decreases with the onset of oocyte maturation and remains low until the onset of the embryonic genome at the 2-cell stage. Heat-induced synthesis of HSP68 and HSC70 does not occur until the morula or blastocyst stage. At the blastocyst stage, but not the 1-cell stage, thermotolerance to a severe heat shock can be induced by prior exposure to a mild heat shock. Further elucidation of the ontogeny of induced thermotolerance has not been described. At the morula stage and probably later antioxidants can serve as thermoprotectants. However, the use of antioxidants as thermoprotectants at earlier stages of development has not been determined.

et al., 1984; Muller *et al.,* 1985; Hahnel *et al.,* 1986). Stem cells were able to synthesize HSP70 molecules in response to heat shock only after undergoing differentiation to inner-cell mass or trophectoderm-like cells (Wittig *et al.,* 1983; Morange *et al.,* 1984). Taken together, these studies suggest that HSP70 proteins become heat-inducible at the morula to blastocyst stage. Since HSP68 and HSC70 have been observed to confer thermal resistance in many cell types (Johnson and Kucey, 1988; Riabowol *et al.,* 1988; Angelidis *et al.,* 1991; Hendrey and Kola, 1991; Li *et al.,* 1991), these HSPs may be responsible, at least in part, for increased resistance to heat shock as murine embryos proceed through development.

Heat-induced production of HSP70 is probably not the only mechanism by which embryos gain resistance to heat shock, because the increase in resistance to heat shock that occurs between the 1-cell and 2-cell stage (Alliston *et al.,* 1965; Gwasdauskas *et al.,* 1992) occurs before the embryo can undergo heat-induced HSP70 synthesis. One possibility is that thermal resistance of murine 2-cell embryos involves constitutive production of HSP70. Protein synthesis from the time of resumption of meiosis in oocytes until the 2-cell stage in mouse embryos is controlled by maternally derived mRNA molecules and not by transcription from the cellular genome (Flach *et al.,* 1982; Bolton *et al.,* 1984). HSP68 and HSC70 represent some of the first constitutive proteins synthesized following genome activation (Bensaude *et al.,* 1983; Manejwala *et al.,* 1991). Since activation of the embryonic genome and constitutive production of HSP68 and HSC70 is coincidental with increased thermal resistance in mouse 2-cell embryos, it is possible that increased thermal resistance in embryos

of other species also occurs at the time of genome activation. Results of Hendrey and Kola (1991) support this hypothesis because microinjection of mRNA for HSC70 to murine oocytes increased resistance to heat shock.

It is also possible that other HSPs are involved with conferring thermal resistance in embryos. In murine embryonic stem cells, heat-induced synthesis of HSP110, HSP90 and HSP60 occurs (Lindquist and Craig, 1988; Burel *et al.,* 1992). HSP27 synthesis may also be heat-induced in these cells, although such synthesis could not be observed through labelling with radiolabelled methionine (Kim *et al.,* 1983b; Arrigo and Welch, 1987). Any or all of these proteins may regulate thermal sensitivity in embryos during early development. HSP90 and HSP27 are likely candidates because these proteins have been reported to increase thermal resistance in other cell types (Landry *et al.,* 1989; Bansal *et al.,* 1991).

Other thermoprotective mechanisms. The intracellular antioxidant, GSH, may also play a role in thermal resistance of embryos. Concentrations of GSH are very high in murine and porcine oocytes, ranging from 1 to 15 mM (Calvin *et al.,* 1986; Yoshida *et al.,* 1993). Aréchiga *et al.* (1992) reported that intracellular GSH was essential for induction of thermotolerance in murine morulae. Induction of thermotolerance was abolished by depletion of intracellular GSH and resistance to heat shock was enhanced by increasing intracellular GSH concentrations prior to heat shock. These observations of Aréchiga *et al.* (1992) also imply that free radical production is increased during embryonic heat shock. Consequently, administration of antioxidants may prove to be a useful method for protecting embryos from heat

shock. In murine morulae, administration of taurine (Malayer *et al.,* 1992), alanine (Malayer *et al.,* 1992) and vitamin E (Aréchiga *et al.,* 1994a) partially prevented detrimental effects of heat shock on embryonic viability.

Strategies for Limiting Deleterious Effects of Heat Stress on Embryonic Survival in Cattle

Studies in the cow (Dunlap and Vincent, 1971; Putney *et al.,* 1988a; Putney *et al.,* 1989a), sheep (Dutt, 1963) and pig (Tompkins *et al.,* 1967; Omtvedt *et al.,* 1971) have determined that the maturing oocyte and early developing embryo are highly sensitive to heat stress effects. It may be possible, therefore, to improve pregnancy rates in heat-stressed cattle by limiting heat stress effects on the maturing oocyte and early developing embryo. Several schemes have been utilized to accomplish this task.

Long-Term Cooling

Solar radiation can be a major contributor of heat to the heat-stressed cow and this heat flow can be reduced by provision of shade. Body temperatures were lower in cows given access to shade structures during heat stress (Roman-Ponce *et al.,* 1977; Buffington *et al.,* 1981; Gwasdauskas *et al.,* 1981; Roman-Ponce *et al.,* 1981). In one study (Roman-Ponce *et al.,* 1977), pregnancy rates were increased from 25.3% to 44.4% by provision of shade. In addition to shade, heat loss may be increased in cattle through convective systems that increase heat transfer from the cow to the surrounding. Rectal temperatures were decreased and pregnancy rates and milk yield were increased by exposure to forced ventilation and shade (Folman *et al.,* 1979; Berman *et al.,* 1985).

Convective heat loss decreases as dry bulb temperature increases (Berman and Meltzer, 1973), and at high temperatures a majority of heat loss occurs through evaporative heat exchange (McLean, 1963). Flamenbaum *et al.* (1986) found that homeothermy was maintained for cows exposed to repeated sprinkling (20 to 30 sec) and forced ventilation (3 to 5 min). Pregnancy rates were improved from 17% to 59% with exposure to repeated cooling for the first 150 d of lactation (Wolfenson *et al.*, 1988). Similarly, pregnancy rates were increased for cattle housed in an air-conditioned facility (Stott *et al.*, 1972; Thatcher *et al.*, 1974). Systems such as these, therefore, are effective in limiting heat stress effects on reproduction in dairy cattle.

Short-Term Cooling

A more economical scheme by which pregnancy rates may be increased during heat stress is provision of cooling for limited periods during early embryonic development. In one such study (Gauthier, 1983), there was a large increase in pregnancy rates for heat-stressed cows cooled for the first 10 d following breeding (13 to 53% pregnant), although low numbers of cows were used (n = 15). Stott and Wiersma (1976) found a marginal increase in pregnancy rates by cooling cows for the first 4 to 6 d following breeding (22 to 30% pregnant) and Her *et al.* (1988) found no benefit to cooling cows from 1 d before until 8 d following breeding. In this study, however, the difference in body temperature between controls and treated cows was small (38.5 to 39.4 C). In summary, short-term cooling is a possible method for improving pregnancy rates during heat stress but further investigation is needed to optimize features that determine its success. Perhaps short-term cooling would be

more successful if cows were also cooled for 2 to 3 d before breeding, when final oocyte maturation occurs.

Embryo Transfer

Pregnancy rates may also be improved during periods of heat stress by embryo transfer. Transfer of embryos on d 6 to 7 after estrus may result in a bypass of detrimental effects of heat stress on oocyte maturation and embryo development. Putney *et al.* (1989b) observed that pregnancy rates of lactating dairy cows exposed to heat stress conditions were higher for recipients of high quality morula- or blastocyst-stage embryos on d 7 postestrus than for cows bred by artificial insemination. Additionally, no depression in pregnancy rates were observed for lactating and nonlactating embryo transfer recipients throughout summer months in the Southwest United States (Putney *et al.,* 1988c).

Manipulation of Embryonic HSP Synthesis

A third approach is to protect the developing embryo from direct effects of heat stress. Manipulation of HSP concentrations within oocytes and embryos may be one way to increase resistance to heat. HSP70, HSP90 and HSP27 have been reported to confer thermal resistance in a number of cells (Johnson and Kucey, 1988; Riabowol *et al.,* 1988; Angelidis *et al.,* 1991; Li *et al.,* 1991). Resistance to heat shock also was increased in murine oocytes with microinjection of mRNA for HSC70 (Hendrey and Kola, 1991). For such manipulations to be used for improving pregnancy rates during heat stress, however, gene expression must be altered. This technology is presently not available for commercial use.

Thermoprotection with Antioxidants

Effects of elevated temperatures may also be minimized by supplementation of antioxidants to decrease damage caused by free radicals during heat stress (Loven, 1988). Effects of heat shock have been reduced in various cultured cells by provision of GSH, taurine or alanine (Vidair and Dewey, 1987; Lumpkin *et al.,* 1988; Kapiszewska and Hopwood, 1988; Malayer *et al.,* 1992). Additionally, murine embryos gained resistance to heat shock effects with administration of alanine (Malayer *et al.,* 1992), taurine (Malayer *et al.,* 1992) and vitamin E (Aréchiga *et al.,* 1994a). In order for such a system to be successful, it will be necessary to identify antioxidants that are effective in preventing effects of heat stress during early stages of development, when embryos are highly sensitive to heat stress effects. It is also necessary to develop delivery systems that increase concentrations of antioxidants in the oviduct sufficient to achieve protection.

Summary

Deleterious effects of heat stress on reproductive performance may be exerted through alterations in a plethora of reproductive processes, including follicular development, oogenesis, spermatogenesis, preimplantation and postimplantation embryonic development, uterine and oviductal function, and hormonal patterns. The maturing oocyte and early developing embryo appear to be particularly susceptible to heat stress. Exposure of cattle to heat stress conditions during final oocyte maturation (Putney *et al.,* 1989b) or for the first 3 to 7 d of pregnancy (Dunlap and Vincent, 1971; Putney *et al.,* 1988a) severely decreased embryonic development and

viability. However, after the first few d of pregnancy, pregnancy rates were less affected by maternal heat stress. Transfer of embryos to lactating dairy cows at d 7 after estrus improved pregnancy rates during heat stress and reduced seasonal variation in pregnancy rate (Putney *et al.,* 1988c; Putney *et al.,* 1989b). In sheep, changes in embryonic resistance to heat stress have been described in detail through experiments that involve exposure of females to heat stress for a single day during early pregnancy (Dutt, 1963). Embryonic viability and development were lower when heat stress occurred on d 0 (breeding) or d 1 of pregnancy than when heat stress occurred on d 3, 5 or 7 of pregnancy. Similar experiments have not been performed in cattle.

Filling gaps in information about developmental changes in embryonic sensitivity to heat may result in development of management schemes to improve pregnancy rates during periods of heat stress in cattle through provision of maximum cooling during critical periods of early pregnancy. Deleterious effects of heat stress can be limited by provision of cooling systems. For example, Wolfenson *et al.* (1988) found that provision of shade, sprinklers and forced ventilation for the first 150 d of lactation during summer months in Israel improved pregnancy rates to levels observed during of winter months. It may be more practical under certain situations to provide maximum environmental modification for only a limited time during early embryonic development. Such an approach has resulted in large (Gauthier, 1983), small (Stott and Wiersma, 1976) or no (Her *et al.,* 1988) beneficial effect on pregnancy rates of heat-stressed cows. This type of system, called strategic cooling

by Hansen *et al.* (1992), might be made more effective if a better understanding of critical periods of embryonic sensitivity to heat stress was acquired.

While it has been shown in several mammals that embryos become more resistant to heat stress as development progresses, there is no information for any species as regards the cellular and biochemical basis for this phenomenon. Resistance of many cultured cells to heat shock can be improved by prior exposure to a mild heat shock (Gerner and Schneider, 1975; Henle and Leeper, 1976; Li and Werb, 1982; Mirkes, 1987; Welch and Mizzen, 1988; Li and Mak, 1989; Maytin *et al.,* 1990; Hatayama *et al.,* 1991). This phenomenon, called thermotolerance, is associated with changes in synthesis of HSPs (Li and Werb, 1982; Li, 1985; Mivechi and Li, 1985; Widelitz *et al.,* 1987; Mizzen and Welch, 1988) and antioxidant molecules (Mitchell *et al.,* 1983; Russo *et al.,* 1984; Jones and Douple, 1990; Harris *et al.,* 1991), which may confer thermal resistance. Establishing the relationship between induced thermotolerance and ontogeny of thermal resistance in mammalian embryos, and identification of specific intracellular processes involved, may lead to development of novel approaches to increase fertility of heat-stressed embryos through biochemical modification of the embryo.

One biochemical system that may be beneficial to modification to protect embryos from adverse effects of heat stress is the antioxidant system. Loven (1988) proposed that heat shock increases free radicals in cells, which then causes cellular damage. Various antioxidants have been shown to reduce effects of heat shock on various types of cultured cells (Vidair and Dewey, 1987; Lumpkin *et al.,* 1988;

Kapiszewska and Hopwood, 1988; Malayer *et al.,* 1992) and in two studies, preimplantation mouse embryos (Malayer *et al.,* 1992; Aréchiga *et al.,* 1994a). However, further investigation of such antioxidants is needed before this type of an approach can be considered a valid means to protect embryos from heat stress effects.

There were two major objectives of the experiments in this dissertation. The first objective involved characterization of the ontogeny of induced thermotolerance in embryos and exploitation of this information to improve fertility in heat-stressed cows. Specific objectives were to identify the period in early pregnancy when the embryo is most susceptible to heat stress, use cultured bovine embryos to evaluate whether developmental changes in susceptibility to maternal heat stress are due to changes in resistance to elevated temperatures, use mouse embryos to evaluate the ontogeny of induced thermotolerance response and finally to test whether cooling cows during critical periods of early pregnancy improves pregnancy rates of heat-stressed cows. The second major objective was to evaluate the efficacy of providing antioxidants to increase resistance of embryos to heat shock. The approach used cultured bovine and murine embryos to identify beneficial antioxidant molecules, with the long-term goal of testing these molecules for thermoprotective effects under field conditions.

CHAPTER III
DEVELOPMENTAL CHANGES IN EMBRYONIC RESISTANCE TO ADVERSE EFFECTS OF MATERNAL HEAT STRESS IN COWS

Introduction

In hot climates, fertility of dairy cattle is depressed during the summer (Poston *et al.,* 1962; Stott and Williams, 1962; Rosenberg *et al.,* 1977; Badinga *et al.,* 1985; Monty and Racowsky, 1987). Heat generated from metabolic functions associated with lactation, growth, and maintenance are not exchanged readily in hot environments, and cows often become hyperthermic when they are exposed to heat stress. A major source for reduction in embryonic survival induced by heat stress may be adverse effects of elevated body temperatures on developing zygotes and embryos. Exposure of cattle to elevated temperatures during oocyte maturation and ovulation (Putney *et al.,* 1989a) or during the first 3 or 7 d of pregnancy (Dunlap and Vincent; Putney *et al.,* 1988a) decreased embryonic viability and development. In other species, embryos become more resistant to elevated temperature as development progresses. In particular, ovine embryos are most sensitive to deleterious effects of maternal heat stress during the first 2 d of pregnancy but become more resistant to maternal heat stress effects by d 3 to 5 after breeding (Dutt, 1963). The increased resistance may be due to development of biochemical

responses within embryos that limit deleterious effects of elevated temperature, i.e., the heat shock response (Lindquist, 1986; Mirkes, 1987).

The objective of the current study was to test whether embryos from superovulated cows become more resistant to adverse effects of maternal heat stress as embryonic development progresses. Superovulated cows were used to increase the number of embryos examined per cow and thereby to improve the efficiency of the experimental design. Identification of stages at which embryos are most susceptible to heat stress will contribute to the knowledge of developmental changes in embryonic responses to stress and is of practical importance because pregnancy rates during periods of heat stress may be improved by cooling cows during critical periods of early pregnancy.

Materials and Methods

Synchronization of Estrus and Superovulation

The experiment was conducted from June to September over 2 consecutive years at the University of Florida Dairy Research Unit in Hague. Groups of 10 to 20 lactating, nonpregnant Holstein cows (50 to 150 DIM) were housed in a free-stall barn containing fans and sprinklers. Fans operated continuously from 0700 to 2000 h, and sprinklers operated for 3 min at 20-min intervals from 0700 to 2000 h daily. Estrous cycles were synchronized by administration of $PGF_{2\alpha}$ (25 mg; Lutalyse®; Upjohn Co., Kalamazoo, MI) twice at 11-d intervals. From 48 to 96 h after the second injection of $PGF_{2\alpha}$, cows were observed for estrus twice daily. Cows observed in standing estrus were superovulated by administration of 44 mg of pituitary-derived

follicle-stimulating hormone (FSH-P; Schering Corp., Kenilworth, NJ) twice daily on d 10 (14 mg/d), 11 (12 mg/d), 12 (10 mg/d), and 13 (8 mg/d) postestrus (23). On d 12 postestrus, $PGF_{2\alpha}$ (25 mg) was administered twice and cows were inseminated artificially three times at 12-h intervals from the onset of standing estrus (onset of estrus = d 0 of pregnancy). A majority of cows demonstrated standing estrus on the morning of d 14; cows not demonstrating standing estrus by this time were administered 4 mg FSH-P and observed for estrous behavior for an additional 24 h.

Treatments

For yr 1, treatments were heat stress on d 1, 3, 5, or 7 of pregnancy or no heat stress (control). For yr 2, treatments were heat stress on d 1 or 3 or control. To induce heat stress, cows were placed in an unshaded lot from 0800 to 1500 h. If a cow experienced rectal temperatures >42 C, she was removed from the heat stress lot and placed under a shade structure until 1500 h. At all other times, except during milking (0700 and 1600 h for yr 1; 0200, 1000, and 1600 h for yr 2), cows were housed in a free-stall barn to maintain thermoneutral conditions.

Cows were assigned randomly to treatments at the onset of estrus. If environmental conditions on the assigned day of treatment apparently would not be conducive to impose heat stress (for example, rain or cool air temperatures), cows were not placed in the lot but were reassigned randomly to other available treatments.

Environmental measurements (black globe temperature, dry bulb temperature, and relative humidity) were recorded on the day of heat stress at 1200 and 1500 h

in the heat stress lot (both years) and in the free-stall barn (yr 2). Rectal temperatures were measured at 1200 and 1500 h on the day of heat stress for all heat-stressed cows (both years) and for control cows at either d 1 or 3 (yr 2).

Determination of Embryonic Survival

On d 8, uteri were flushed nonsurgically to retrieve embryos and were classified according to stage of development (Drost, 1986). Viability was determined using the vital stain 4',6'-diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, MO). Embryos were incubated in Dulbecco's PBS (DPBS; pH 7.4, 25 C) containing 0.0001% DAPI for 15 to 20 min at room temperature, washed in Dulbecco's PBS and examined using an epifluorescence microscope with a 490-nm emission filter (Schilling *et al.*, 1979). Embryonic viability was scored on a four-point scale according to the proportion of cells within embryos that stained positive for DAPI: DAPI score was 1 when no cells stained, 2 when fewer than one-third of the cells stained, 3 when one- to two-thirds of the cells stained and 4 when more than two-thirds of the cells stained. Embryos were considered to be live when fewer than one-third of the cells stained positive for DAPI (scores of 1 and 2).

Statistical Analysis

Data were analyzed using both categorical procedures (CATMOD) and least squares ANOVA (GLM) through procedures of SAS (1989). One-cell embryos were considered to be unfertilized oocytes and were removed from all analyses except for the stage of embryonic development at d 8 and percentage of embryos at the blastocyst stage. Number of cows and embryos for data including and excluding 1-

cell embryos/unfertilized oocytes are presented in Table 3-1. For categorical procedures (DAPI score, percentage of embryos at each stage of development, and distribution of embryos within stage), the model included components of treatment, year, and treatment x year interactions. Stages of embryonic development included 1-cell, 2- to 8-cells, 9- to 16-cells, morula and blastocyst. Data were analyzed using all treatments (complete data set) and then reanalyzed after excluding treatments on d 5 and 7 (reduced data set), since these treatments were not represented during yr 2. For ANOVA (rectal temperature, DAPI score, percentage of live embryos, and percentage of blastocysts), cow was used as the experimental unit for effects of treatment, year, treatment x year, and experiment(treatment x year). These analyses were accomplished by analysis of average embryonic responses for each cow. Preplanned orthogonal contrasts were performed to separate treatment effects and consisted of control, d 1 and 3 versus d 5 and 7; d 5 versus 7; control and d 3 versus d 1; and control versus d 3. For the reduced data set, contrasts were control and d 3 versus d 1; and control versus d 3. For rectal temperature, Duncan's multiple range test was used to evaluate differences among treatments.

Results

Environmental Conditions and Rectal Temperatures

Peak black globe temperature, dry bulb temperature, and relative humidity in the unshaded lot averaged 42.1 C, 34.3 C and 53.7% for yr 1 and 41.5 C, 34.7 C and 64.4% for yr 2, respectively. During yr 2, black globe temperatures, dry bulb temperatures, and relative humidity in the control environment averaged 31.8 C, 30.5

C and 64.4%, respectively. Rectal temperatures were higher ($P \leq 0.05$) for cows placed in the unshaded lot than for controls (40.9 to 41.7 versus 39.1 C; Table 3-1) and are comparable to rectal temperatures observed in previous studies (Putney *et al.*, 1988a; Putney *et al.*, 1989a). Variation in rectal temperatures among cows was not a significant factor in embryonic responses since use of rectal temperature as a covariate did not affect results.

Embryonic Survival

A CATMOD analysis revealed an effect of treatment on DAPI score when all data were analyzed ($P = 0.07$) or after embryos from cows heat stressed on d 5 and 7 were excluded ($P = 0.03$). As shown in Figure 3-1, maternal heat stress on d 1 resulted in fewer embryos with DAPI scores of 1 and 2 and more embryos with DAPI scores of 4. Least squares means for DAPI scores are presented in Table 3-1. As determined by ANOVA of orthogonal contrasts, DAPI score was greater for embryos from cows heat stressed on d 1 than for embryos from control cows and cows heat stressed on d 3 (complete data set, $P = 0.07$; reduced data set, $P = 0.05$). Day of heat stress also affected the proportion of embryos classified as live or dead based on CATMOD analysis of DAPI scores (Table 3-1; $P = 0.03$ for complete and reduced data sets). The percentage of live embryos was also calculated for each cow, and data were analyzed by least squares ANOVA to consider variation among cows in the analysis. Least squares means for the percentage of live embryos were 70.9% for embryos from control cows and 54.9, 60.3, 62.6, and 82.0% for embryos from cows heat stressed on d 1, 3, 5, and 7, respectively ($P = 0.07$, d 1 versus control and d 3

Table 3-1. Effects of maternal heat stress for one day during early pregnancy on rectal temperature and embryonic viability.

Day of heat stress	Cows[a] (≥1-cell embryos)	Embryos (≥1-cell embryos)	Cows[b] (≥2-cell embryos)	Embryos (≥2-cell embryos)	Peak rectal temperature[c]	Mean DAPI score[d]	Live embryos[e]
	(n)				(C)		(%)
Control	20	118	18	94	39.1	2.2	70.2
1	11	50	9	40	41.3	2.5	55.0
3	8	50	8	50	40.9	2.0	68.0
5	10	23	9	20	41.7	2.2	65.0
7	5	31	5	27	41.0	1.7	88.9

[a]Cows from which at least one embryo at 1-cell (unfertilized oocyte) or subsequent stages were retrieved.

[b]Cows from which at least one embryo at 2-cell or subsequent stages were retrieved.

[c]Least squares means (SEM = 0.08 C). Controls differed from other treatments ($P \leq 0.05$; Duncan's multiple range test.

[d]Least squares means (SEM = 0.11). CATMOD analysis demonstrated an effect of treatment on 4',6'-diamidino-2-phenylindole (DAPI) score ($P = 0.07$ for complete data set, $P = 0.03$ for reduced data set). By ANOVA, DAPI score differed for embryos from cows heat stressed on d 1 compared with embryos from control cows and cows heat stressed on d 3 ($P = 0.07$ for complete data set, $P = 0.05$ for reduced data set). Contrasts for embryos from control cows versus embryos from cows heat stressed on d 3; embryos from cows heat stressed on d 5 versus embryos from cows heat stressed on d 7; and control, d 1 and d 3 versus d 5 and d 7 were not significant.

[e]Represents percentages calculated from the number of live embryos divided by the total number of embryos (≥2-cell). By CATMOD, an effect of treatment on the percentage of live embryos was observed ($P = 0.03$ for complete and reduced data set). By ANOVA, the percentage of live embryos differed on d 1 compared with control and d 3 ($P = 0.10$ for complete data set, $P = 0.07$ for reduced data set). Other orthogonal contrasts (control versus d 3; d 5 versus d 7; and control, d 1 and d 3 versus d 5 and d 7) were not significant.

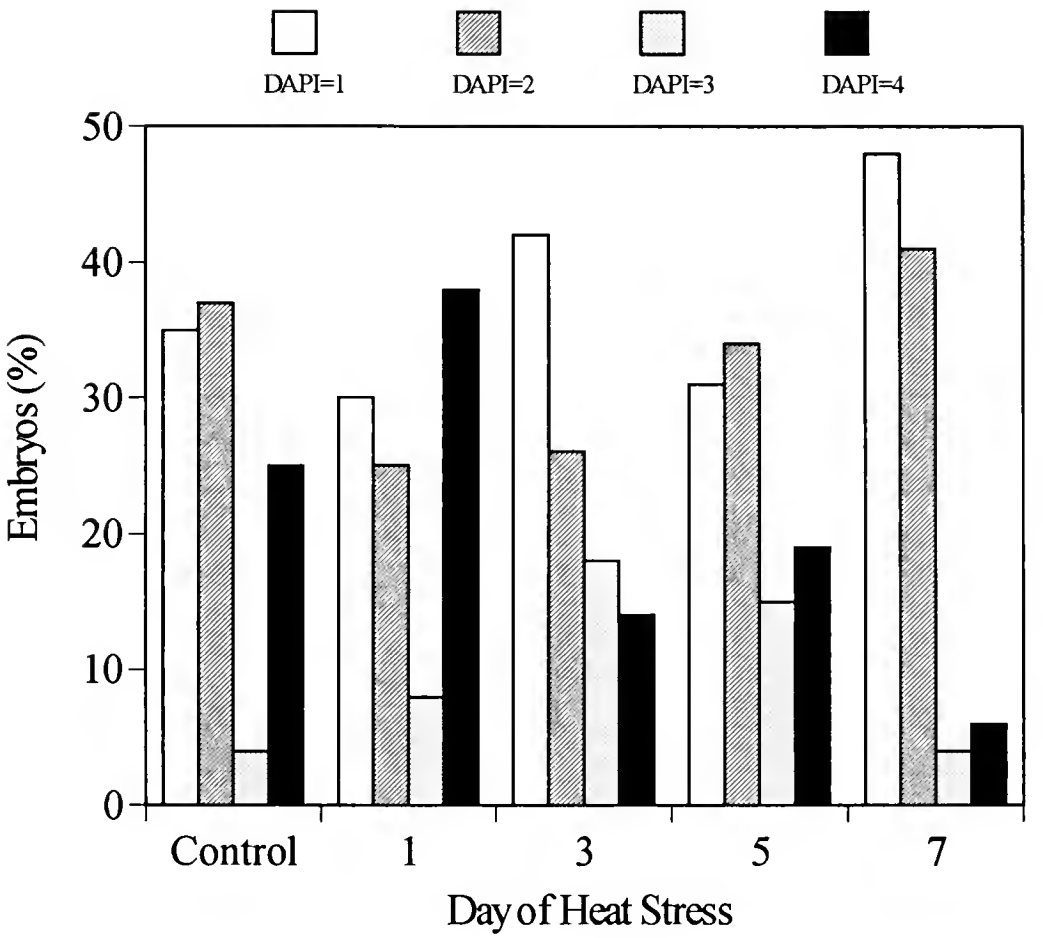

Figure 3-1. Effect of maternal heat stress on 4',6'-diamidino-2-phenylindole (DAPI) score of embryos (≥2-cell). Results represent the percentage of embryos within each treatment with DAPI scores of 1 (no cells stained), 2 (less than one-third of the cells stained), 3 (one-third to two-third of the cells stained) and 4 (more than two-third of the cells stained).

for the reduced data set; SEM = 12%). No year x treatment interactions were detected for any of the models used to analyze mean DAPI score or percentage of live embryos.

Embryonic Development

Alterations in the distribution of stage of embryonic development (Table 3-2) were evident among treatments as determined by CATMOD analysis of all treatments (P = 0.10), or after exclusion of embryos from cows heat stressed on d 5 and 7 (P = 0.03). Individual analysis of the percentage of embryos at each stage showed that heat stress on d 1 decreased the percentage of embryos at the blastocyst stage of development (P = 0.02 for the complete data set, P = 0.05 for the reduced data set) and increased the percentage of embryos at the 9- to 16-cell stage of development (P = 0.04 for the complete data set, P = 0.01 for the reduced data set). In addition, day of heat stress affected percentage of 1-cell embryos/unfertilized oocytes (P = 0.03 for complete data set; P = 0.05 for the reduced data set), being lower for cows treated at d 3. No treatment effects were observed at other stages. Least squares means for the percentage of blastocysts, when 1-cell embryos were removed from the data sets, were 60.6% for embryos from control cows and 37.4, 54.8, 62.6, and 64.5% for embryos from cows heat stressed on d 1, 3, 5, and 7, respectively; the percentage of blastocysts was decreased only on d 1 (P = 0.07 for complete data set, P = 0.06 for reduced data set when results from d 1 were compared with those for controls and d 3; SEM = 13%). When 1-cell embryos/ unfertilized oocytes were included in the analysis, least squares means for the

Table 3-2. Effect of maternal heat stress during early pregnancy on stage of embryonic development on d 8 of pregnancy.

Day of heat stress	Embryos	Embryos				
		1-Cell[a]	2- to 8-Cell	9- to 16-Cell[b]	Morula	Blastocyst[c]
	(n)	(%)				
Control	118	20	11	5	15	49
1	50	20	12	18	16	34
3	50	0	10	6	24	60
5	23	13	4	4	22	57
7	31	13	0	6	17	64

[a]Treatment effect by CATMOD; $P = 0.03$ for complete data set, $P = 0.05$ for reduced data set.

[b]Treatment effect by CATMOD; $P = 0.04$ for complete data set, $P = 0.01$ for reduced data set.

[c]Treatment effect by CATMOD; $P = 0.02$ for complete data set, $P = 0.05$ for reduced data set. By ANOVA, percentage blastocysts were reduced on d 1 compared with control and d 3 ($P = 0.07$ for complete data set, $P = 0.06$ for reduced data set). Other orthogonal contrasts (control versus d 3; d 5 versus d 7; and control, d 1 and d 3 versus d 5 and d 7) were not significant.

percentage of blastocysts were 42.1% for embryos from control cows and 28.4, 54.8, 63.0, and 58.5% for embryos from cows heat stressed on d 1, 3, 5, and 7, respectively. With this analysis, heat stress on d 1 decreased percentage of blastocysts compared to embryos from control cows and from cows heat stressed on d 3 (P = 0.03 for complete data set; P = 0.05 for reduced data set; SEM = 11%). Year x treatment interactions were not significant for any statistical analysis of embryonic development.

Discussion

These results demonstrate that bovine embryos become more resistant to deleterious effects of maternal heat stress as they proceed through development. Thus, the cow is similar to the sheep (Dutt, 1963) and pig (Tompkins *et al.,* 1967; Omtvedt *et al.,* 1971) in this regard. A minimum period after heat stress before evaluation of embryonic viability and development could preclude the ability to detect adverse effects for embryos on d 5 and 7 treatments. Since no effects of heat stress on embryos were observed on d 3, however, it was apparent that embryos become more resistant to heat stress as development progresses. The magnitude of adverse effects of maternal heat stress on embryonic survival was less severe in the present study than for studies in other species. Differences may be attributed to method of heat stress; studies in sheep (Dutt, 1963) and pigs (Tompkins *et al.,* 1967; Omtvedt *et al.,* 1971) were performed in environmentally controlled chambers for 17 to 24 h, whereas the present study was performed under more variable environmental conditions for 7 h. Present findings do not imply that bovine embryos are completely resistant to effects of maternal heat stress by d 3 of pregnancy; heat stress of greater

severity or duration than that used in this study could possibly decrease embryonic survival before or after d 1. Maternal heat stress during final oocyte maturation and ovulation has deleterious effects on subsequent embryonic development that is more severe than was observed in the present study (Putney *et al.,* 1989a). In addition, conceptus development was decreased by exposure of cows to heat stress from d 8 to 16 of pregnancy (Biggers *et al.,* 1987). Nonetheless, these results demonstrate that embryos respond differentially to maternal heat stress depending on their stage of development, and, by d 3 of pregnancy, embryos have acquired some resistance to adverse effects of maternal heat stress.

Mechanisms that are responsible for the ontogeny of embryonic resistance to thermal stress are not defined but could reflect changes in embryonic function or in the microenvironment of the embryo. Embryos may develop the capacity to produce molecules that limit effects of heat on cellular function. In many cells, the synthesis of HSPs during elevated temperature limited deleterious effects of elevated temperatures (Li and Werb, 1982; Riabowol *et al.,* 1988; Landry *et al.,* 1989; Bansal *et al.,* 1991; Hendrey and Kola, 1991). In mouse embryos, thermal resistance was increased for 2-cell embryos compared with 1-cell embryos (Gwasdauskas *et al.,* 1992), coincident with the partial activation of the embryonic genome (Prather and First, 1988) and constitutive synthesis of HSP70 molecules (Bensaude *et al.,* 1983; Manejwala *et al.,* 1991). The development of bovine embryonic resistance to maternal heat stress in cattle may be due to the ability of embryos to produce HSP in response to elevated temperatures. Consistent with this theory is the fact that the

bovine embryonic genome is activated between the 4- to 8-cell stage (Barnes and Eyestone, 1990; Barnes and First, 1991), i.e., d 2 to 3 of pregnancy (Betteridge and Fléchon, 1988).

Developmental resistance of embryos to maternal heat stress may also involve interactions between the embryo and reproductive tract. Heat stress altered protein secretion from the oviduct and uterus (Malayer *et al.,* 1988; Geisert *et al.,* 1988; Putney *et al.,* 1988b; Malayer and Hansen, 1990). The increase in resistance of embryos to heat stress is not likely changed by a shift in the location of embryos from oviduct to uterus because embryos are present in the oviduct at d 1 and 3 of pregnancy (Betteridge and Fléchon, 1988).

This study may have practical implications for improving fertility during summer in hot climates. Hansen *et al.* (1992) proposed that, while cows should receive cooling at all times during heat stress, summer pregnancy rates may be improved during periods of heat stress through strategic cooling, in which cows are placed in an environment that allows maximal cooling during times when embryos are most sensitive to heat stress effects. These times have been defined as the period of final oocyte maturation and ovulation (Putney *et al.,* 1989a) and the first few days of pregnancy (present study). Also, administration of agents that protect embryos from adverse effects of heat stress may be a useful approach to improve pregnancy rates during summer. Taurine, alanine and vitamin E supplementation to medium improved embryonic viability and development in cultured bovine embryos exposed to elevated temperatures (Malayer *et al.,* 1992; Aréchiga *et al.,* 1994a). Present results

indicate that the most beneficial time to administer such agents would be before d 3 of pregnancy.

In conclusion, bovine embryos are sensitive to deleterious effects of maternal heat stress at d 1 of pregnancy but become more resistant to heat stress effects by d 3 of pregnancy. These results suggest that the embryo acquires thermotolerance during development and that improved fertility may be possible during periods of heat stress if cooling is provided for a limited period when embryos are most sensitive to thermal stress.

CHAPTER IV
DEVELOPMENTAL CHANGES IN SENSITIVITY OF CULTURED BOVINE EMBRYOS TO HEAT SHOCK

Introduction

Exposure of cows to elevated dry bulb temperatures during estrus or the first 3 to 7 d of pregnancy greatly decreased embryonic development and viability (Dunlap and Vincent, 1971; Putney *et al.,* 1988a; Putney *et al.,* 1989a). Processes leading to successful pregnancy become less susceptible to disruption by heat stress as pregnancy proceeds. Heat stress at d 1 of pregnancy caused a decrease in embryonic development and survival, whereas heat stress at d 3 of pregnancy or later had no effect (Chapter III). Similar developmental changes in embryonic resistance to maternal hyperthermia have been reported in sheep (Dutt, 1963) and pigs (Omtevedt *et al.,* 1967; Tompkins *et al.,* 1967).

Effects of elevated temperature on early embryos appear to be a major cause for the decrease in pregnancy rates after maternal heat stress. This is so because embryos transferred from heat-stressed ewes to non-heat stressed recipients were less capable of continued development compared with embryos transferred from non-heat stressed ewes into heat-stressed recipients (Alliston and Ulberg, 1961). Additionally, exposure of embryos *in vitro* to elevated temperatures disrupts embryonic development and viability in other mammalian species (Alliston *et al.,* 1965; Aréchiga

et al., 1992; Gwasdauskas *et al.,* 1992; Malayer *et al.,* 1992; Aréchiga *et al.,* 1994a). Currently, it is unclear whether there are developmental changes in embryonic sensitivity to heat shock that could explain results indicating differential maternal responses to heat. In the mouse, there is evidence to indicate that as embryos develop, they acquire the ability to undergo induced thermotolerance response (Muller *et al.,* 1985; Chapter V). Similar information, however, is not available for other species. Therefore, the present studies were conducted to characterize temperatures that disrupt bovine embryonic development *in vitro* and to determine if bovine embryos gain resistance to heat shock as they progress from the 2-cell to morula stage.

Materials

Bovine steer serum (BSS) was purchased from Pel-Freez (Rogers, AR), FSH-P was from Schering Corp. (Kenilworth, NJ), frozen semen from various Holstein bulls was obtained from American Breeders Service (Madison, WI) and Buffalo rat liver cells (line BRL-3A) were obtained from American Type Culture Collection (Rockville, MD). CZB medium was prepared as described by Chatot *et al.* (1989) and modified Tyrode's solutions (HEPES-TALP and IVF-TALP) was prepared as described by Parrish *et al.* (1986). Ovary transport solution was prepared by addition of 550 μg/L amphotericin, 100,000 IU/L penicillin-G and 100 mg/L streptomycin in 0.9% (w/v) NaCl.

Methods

In Vitro Maturation/Fertilization/Culture

The procedures used to produce *in vitro* derived embryos, which are outlined in detail in Appendix A, were modifications of existing procedures (Parrish *et al.,* 1988; Xu *et al.,* 1992; Hernandez-Ledezma *et al.,* 1993). In brief, bovine ovaries were obtained from a local abattoir and transferred to the laboratory in sterile transport solution at 23 to 26 C. Ovaries were washed repetitively in fresh saline upon arrival and placed in a warm room (~30 C) for oocyte collection. Checkerboard incisions (~2 mm depth) were then made on the surface of each ovary using scalpel blades. Ovaries were washed vigorously in collection medium [TCM-199 with Hank's salts, 10 mM Hepes, 2% (v/v) BSS, 40 units/L heparin and 1% (v/v) ABAM]. Oocytes containing at least one complete layer of surrounding cumulus cells and evenly granulated ooplasm were placed (~300 oocytes/10 ml medium in 60 x 15 mm culture dishes) in maturation medium [TCM-199 with Earle's salts, 10% (v/v) BSS, 2 μg/ml estradiol, 20 μg/ml FSH-P and 50 μg/ml gentamicin] and were incubated at 39 C in an atmosphere of 5% CO_2 in air for 24 to 26 h. An average of 11.5 oocytes were collected per ovary.

Following maturation, oocytes containing expanded cumulus were washed in HEPES-TALP and placed in IVF-TALP (~30 oocytes/600 μl medium in 4-well plates). Viable spermatozoa were recovered from frozen/thawed semen of 2 to 4 bulls by Percoll gradient centrifugation (Hernandez-Ledezma *et al.,* 1993) and added to IVF-TALP at a final concentration of 1 x 10^6 spermatozoa/ml medium.

Immediately following addition of spermatozoa, epinephrine (40 μM), hypotaurine (80 μM), and penicillamine (10 μM) were added to wells to improve fertilization rate (Leibfried and Bavister, 1982). Oocytes were then incubated at 39 C for 16 to 18 h (d 0 post-IVF = day of fertilization). Cleavage rate averaged 47.6% and rate of parthenogenesis averaged 5.7%.

At 16 to 18 h following fertilization (d 1 post-IVF), zygotes were removed from wells, vortexed to remove cumulus cells and attached spermatozoa, and washed in HEPES-TALP. Zygotes were then placed either in 600 μl CZB medium containing 10% (v/v) BSS and 50 μg/ml gentamicin or in 50 μl microdrops of TCM-199 medium with Earle's salts, 10% (v/v) BSS and 50 μg/ml gentamicin covered in paraffin oil. Before use in embryo culture, both media were conditioned for 24 to 48 h by Buffalo rat liver cells. Conditioned medium was sterile-filtered and stored for up to 1 wk at 4 C.

Effects of Heat Shock on 2-cell Embryos

Putative embryos were collected at d 1 post-IVF (~50) and placed in wells containing 600 μl modified CZB medium for 18 to 24 h at 39 C in 5% CO_2. On d 2 post-IVF, 2-cell embryos were placed in groups of 15 to 25 into wells containing 600 μl modified CZB medium, incubated for 1 h at 39 C, and exposed to heat shock treatments. Following heat shock, embryos were placed at 39 C. Embryos were transferred to 600 μl modified TCM-199 on d 3 post-IVF and then incubated at 39 C until d 5 post-IVF, when development was assessed.

Effects of Heat Shock on Morula Stage Embryos

Putative embryos on d 1 post-IVF (~20) were placed into 50 μl drops of modified TCM-199 covered with paraffin oil and cultured at 39 C. On d 3 post-IVF, an additional 50 μl of modified TCM-199 was added to each drop. Morulae were collected on d 5 post-IVF and groups of 9 to 15 morulae were placed either in 50 μl drops of modified CZB medium or modified TCM-199 medium, placed at 39 C for 1 h, and exposed to heat shock treatments. Following heat shock, embryos were maintained at 39 C. For embryos placed in modified CZB medium on d 5 post-IVF, embryos were transferred to modified TCM-199 microdrops on d 6 post-IVF. Embryos placed in modified TCM-199 medium on d 5 post-IVF were maintained in the same microdrop for the duration of culture. Stage of development was recorded on d 9 post-IVF. Data from CZB and TCM-199 cultures were pooled for analysis since the proportion of embryos that developed to the blastocyst stage was not affected by type of medium used on d 5 post-IVF.

Statistical Analysis

Each experiment was performed on several different days, using one or more wells or microdrops (replicates) of embryos per treatment on each day. Treatment effects on the number of embryos undergoing development in each replicate (*i.e.*, each well or microdrop) were analyzed by ANOVA (SAS, 1989). Class effects in the model were temperature, day and temperature x day. The total number of embryos in each replicate was used as a covariate in these analyses. Orthogonal contrasts were performed to partition treatment effects into individual comparisons (SAS,

1989). Probability values represent results from these analyses. To calculate least squares means for percentage of embryos undergoing development, percent development for each replicate were also analyzed by ANOVA. Each data set was tested for heterogeneity of variance; when present (experiment 1 only), individual standard errors were calculated for each treatment. Otherwise, standard errors generated from ANOVA were reported.

Results

In the first experiment, 2-cell embryos were exposed to heat shocks of 40 C, 41 C or 42 C for 3 h (Table 4-1). Compared with controls incubated continuously at 39 C, exposure to 40 C did not influence the proportion of embryos that developed to ≥16-cell stages on d 5 post-IVF. However, heat shock of 41 C or 42 C greatly decreased subsequent development (P = 0.004) compared with embryos cultured at 39 or 40 C. Sensitivity of 2-cell embryos to heat shock was further determined by exposing 2-cell embryos to a 41 C heat shock for 1 or 3 h (Table 4-2). Compared with embryos cultured at 39 C continuously, exposure to 41 C for 3 h decreased (P = 0.01) subsequent development of 2-cell embryos, whereas heat shock of 41 C for 1 h did not affect development. Effects of a 41 C heat shock were also determined for morula stage embryos (Table 4-2). In contrast to embryos at the 2-cell stage, there was no effect of 41 C for 1 or 3 h on subsequent development.

Discussion

Exposure of 2-cell bovine embryos to a heat shock of as little as 41 C for 3 h decreased embryonic development as determined on d 5 post-IVF. This

Table 4-1. Effect of exposure of 2-cell bovine embryos to various temperatures on subsequent embryonic development.

Treatment	Number of Replicates	Number of Embryos	Percent Embryos Developing to ≥16-cell Stage*
39 C	6	109	31.4 ± 5.8[a]
40 C - 3 h	6	110	29.2 ± 8.3[a]
41 C - 3 h	6	107	10.6 ± 3.4[b]
42 C - 3 h	6	97	2.6 ± 1.8[b]

*Data represent least-squares means ± SEM of embryonic development on d 5 post-IVF. Means with different superscripts differ as determined by orthogonal contrasts between 39 C versus 40 C (Not Significant), 41 C versus 42 C (Not Significant), and 39 C and 40 C versus 41 C and 42 C (P = 0.004). The probability for an overall temperature effect was P = 0.01.

Table 4-2. Effects of a 41 C heat shock on subsequent development of 2-cell and morula stage bovine embryos.

Treatment	Number of Replicates	Number of Embryos	Percent Embryos Undergoing Subsequent Development*
2-cell			
39 C	6	138	33.2 ± 4.2[a]
41 C - 1 h	6	137	27.3 ± 4.2[a]
41 C - 3 h	6	138	12.2 ± 4.2[b]
Morula			
39 C	6	65	30.0 ± 4.9[a]
41 C - 1 h	6	64	22.8 ± 4.9[a]
41 C - 3 h	6	67	21.7 ± 4.9[a]

*Data represent least-squares means ± SEM for percentage of 2-cell embryos developing to ≥16-cell stages on d 5 post-IVF or percentage of morula developing to blastocysts on d 9 post-IVF. Within each stage of development, different superscripts designate differences determined by orthogonal contrasts. For 2-cell embryos, differences were observed when comparing 41 C for 1 h versus 41 C for 3 h (P = 0.01) but not when comparing 41 C for 1 h and 41 C for 3 h versus 39 C. No differences were observed for contrasts at the morula stage. Overall, heat shock decreased subsequent development of 2-cell embryos (P = 0.03) but not morulae.

temperature is similar to body temperatures achieved in cattle during periods of heat stress (Putney *et al.,* 1988a; Putney *et al.,* 1989a; Chapter III), suggesting that a prominent cause of decreased embryonic survival from maternal heat stress is the direct effect of elevated body temperature on embryonic development and survival. In contrast to embryos at the 2-cell stage, morulae were not affected by heat shock of 41 C for 1 or 3 h. Thus, bovine embryos seem to acquire increased thermal resistance during embryonic development. It is possible that embryos which possess thermal resistance capabilities were selected for during *in vitro* development. However, the change in thermal resistance with increased development *in vitro* agrees closely with previous observations *in vivo.* In particular, deleterious effects of maternal heat stress on embryonic survival were less on d 3 of pregnancy than on d 1 of pregnancy in cattle (Chapter III) and sheep (Dutt, 1963).

Although mechanisms responsible for the developmental resistance of embryos to heat shock are not defined in cattle, the development of embryonic resistance to heat shock may be determined by when heat-inducible changes in synthesis of HSPs or other thermoprotective molecules occurs. Constitutive synthesis of HSP70 molecules includes some of the first proteins synthesized by the mouse embryo upon genome activation (Bensaude *et al.,* 1983; Manejwala *et al.,* 1991). If the cow embryo is similar, then the absence of embryonic genome expression of HSPs before the 4- to 8-cell stage (Barnes and Eyestone, 1990; Barnes and First, 1991) may cause increased sensitivity to elevated temperatures. The period of embryonic sensitivity to heat shock may be extended further because mouse embryos lack the ability to

produce HSP70 in response to heat during initial cleavage stages, whereas production is possible at later stages (Wittig *et al.,* 1983; Morange *et al.,* 1984; Muller *et al.,* 1985; Hahnel *et al.,* 1986). Future experiments will be required to determine whether the acquisition of thermal resistance in bovine embryos coincides with activation of HSP synthesis or expression of additional thermoprotective mechanisms in response to heat. Increased thermal sensitivity of embryos during initial cleavage stages may also be due to the potentially more deleterious effect of loss of a few blastomeres caused by heat shock in early embryos than embryos at later stages of development.

CHAPTER V
INDUCED THERMOTOLERANCE DURING EARLY DEVELOPMENT OF MURINE AND BOVINE EMBRYOS

Introduction

It has been established that during initial stages of development, embryos are sensitive to elevations in temperature both *in vivo* (Chapter III) and *in vitro* (Chapter IV). As development progresses, however, embryos are less affected by elevated temperature. This observation has been shown in cattle (Chapter III; Chapter IV), sheep (Dutt, 1963), pigs (Tompkins *et al.,* 1967; Omtvedt *et al.,* 1971), mice (Gwasdauskas *et al.,* 1992) and rabbits (Alliston *et al.,* 1965). It is postulated that the sensitivity of maturing oocytes and early developing embryos to elevated temperature is due to the lack of biochemical mechanisms that confer resistance to heat.

While cell proliferation, protein synthesis and viability are compromised upon exposure to heat shock, cells can undergo biochemical processes to limit these deleterious effects. These processes are responsible for a phenomenon termed induced thermotolerance, in which effects of severe heat shock are reduced by prior exposure to a less severe heat shock (Gerner and Schneider, 1975; Henle and Leeper, 1976; Li and Werb, 1982; Mirkes, 1987; Mizzen and Welch, 1988; Welch and Mizzen, 1988; Li and Mak, 1989; Maytin *et al.,* 1990; Hatayama *et al.,* 1991). Among

the biochemical changes in cells responsible for induction of thermotolerance is production of HSPs. These proteins, which are produced in increased amounts in response to heat shock, have been implicated in protecting heat shocked cells by inhibiting protein synthesis, refolding damaged proteins and protecting ribosomal RNA (Lindquist, 1986; Nover and Scharf, 1991). Proteins in the HSP70 family, which include the constitutively expressed form (HSC70 in the mouse; Giebel *et al.,* 1988) and heat-inducible form (HSP68 in the mouse; Hunt and Calderwood, 1990), have been implicated in eliciting thermotolerance to cells. Microinjection of mRNA for the human HSC70 induced thermotolerance in fibroblasts (Li *et al.,* 1991) and murine oocytes (Hendrey and Kola, 1991), and removal of HSP70 proteins by antibody treatment prevented induction of thermotolerance (Riabowol *et al.,* 1988). Hence, HSP70 molecules are an essential component for the induction of thermotolerance in cells.

In contrast to most cells, maturing oocytes and early developing embryos lack the ability to produce increased amounts of HSPs in response to heat shock. In the mouse, levels of HSP68 and HSC70 protein (Curci *et al.,* 1987; Curci *et al.,* 1991) and HSC70 messenger RNA (Manejwala *et al.,* 1991) decrease greatly after germinal vesicle breakdown. Constitutive production of HSP68 and HSC70 protein begins at the 2-cell stage, but heat-inducible synthesis of these proteins has not been observed until the morula to blastocyst stage (Wittig *et al.,* 1983; Morange *et al.,* 1984; Muller *et al.,* 1985; Hahnel *et al.,* 1986). Therefore, lack of heat-inducible production of HSPs or other biochemical changes causing thermotolerance during oocyte

maturation and early embryonic development may predispose sensitivity to elevated temperatures. The following experiments investigate this hypothesis by determining the ontogeny of induced thermotolerance in preimplantation mouse embryos. Murine embryos were used as a model for investigating the ontogeny of thermal resistance in mammalian species because of previous research involving the ontogeny of HSP synthesis. An additional objective was to determine whether induced thermotolerance could be demonstrated in bovine embryos.

Materials and Methods

Mouse Superovulation, Embryo Recovery and Culture

Mice (ICR outbred strain; Harlan Sprague Dawley Inc., Indianapolis, IN) were housed at 25 C in 14 h light and 10 h dark photoperiod cycles with 2400 h as the midpoint of the dark cycle. Procedures used for collection and culture of mouse embryos is described in Appendix B. Female mice (21 to 30 d of age) were superovulated by an intraperitoneal injection of pregnant mare serum gonadotropin (PMSG; 10 IU; Sigma Chemical Co.) followed 44 to 46 h later with human chorionic gonadotropin (hCG; 7.5 IU; Sigma Chemical Co.). Females were placed with fertile males overnight and coitus was determined the following morning by presence of a vaginal plug. In some experiments, females were sacrificed 24 h to 36 h after observation of vaginal plugs (2-cell to 4-cell stage), oviducts were dissected, and oviducts were flushed with M2 medium (Quinn *et al.,* 1982) containing 0.4% bovine serum albumin (BSA; Fraction V, 96 to 99% pure; Sigma Chemical Co.) using a 30 ga. needle. Five to ten embryos were then placed in 5 to 7 μl of M16 medium

containing 0.4% BSA (Whittingham, 1971), covered with double-extracted, sterile-filtered silicon oil (Aldrich Chemical Co. Inc., Milwaukee, WI) and incubated at 37 C (5% CO_2 in air; approximately 70% relative humidity) until the desired stage of development. These embryos will subsequently be referred to as embryos developed *in vitro*.

In other experiments, females were sacrificed 50 h (8-cell), 60 h (early morula) or 72 h (blastocyst) after observation of vaginal plugs. Oviducts or uterine horns were dissected and flushed with M2 medium containing 0.4% BSA. Embryos were immediately used in experiments; consequently, these embryos will be referred to as embryos developed *in vivo*.

Cow Superovulation, Embryo Recovery and Culture

Estrous cycles of lactating Holstein cows were synchronized with intramuscular administration of $PGF_{2\alpha}$ (25 mg) twice at 11 d intervals. Cows observed in estrus were superovulated with twice daily injections of FSH-P (44 mg) from 10 to 14 d following observation of estrus. Prostaglandin $F_{2\alpha}$ was administered on d 12 after estrus. At the subsequent estrus, cows were artificially inseminated three times at 12 h intervals beginning at the onset of estrus. Eight days after the first breeding, each uterus was flushed nonsurgically and blastocysts retrieved were placed in 500 μl modified Hams-F10 medium (10% htFCS, 100,000 IU/L penicillin G and 100 mg/L streptomycin).

Induction of Thermotolerance

At desired stages of development, mouse embryos developed *in vitro* or *in vivo* were placed in 5 to 7 μl of M16 medium containing 10% htFCS or 0.4% BSA and incubated at 37 C for 1 h. Cow embryos were placed in 500 μl modified Hams-F10 medium and incubated at 38.5 C for 1 h. Embryos were then exposed to one of the heat treatments depicted in Figure 5-1. For some experiments (Figure 5-1a), the severe heat shock used was exposure to 43 C for 2 h. The four treatments imposed were 37 C for 5 h (control); 40 C for 1 h and 37 C for 4 h (mild heat shock); 37 C for 3 h and 43 C for 2 h (severe heat shock); or 40 C for 1 h, 37 C for 2 h and 43 C for 2 h (to induce thermotolerance). These heat treatments were designated as 37 C, 40 C, 43 C and 40/43 C, respectively. In other experiments (Figure 5-1b), a severe heat shock treatment of 42 C for 1 h replaced the heat shock of 43 C for 2 h. Embryos in these experiments were exposed to heat treatments of 37 C for 5 h; 40 C for 1 h and 37 C for 4 h; 37 C for 3 h, 42 C for 1 h and 37 C for 1 h (42 C treatment); or 40 C for 1 h, 37 C for 2 h, 42 C for 1 h and 37 C for 1 h (40/42 C treatment). Bovine embryos were exposed to heat shock treatments depicted in Figure 5-1a, except that 38.5 C, instead of 37 C, was used as the control temperature. Following both series of heat treatments, murine and bovine embryos were incubated at 37 C and 38.5 C, respectively, for 19 h.

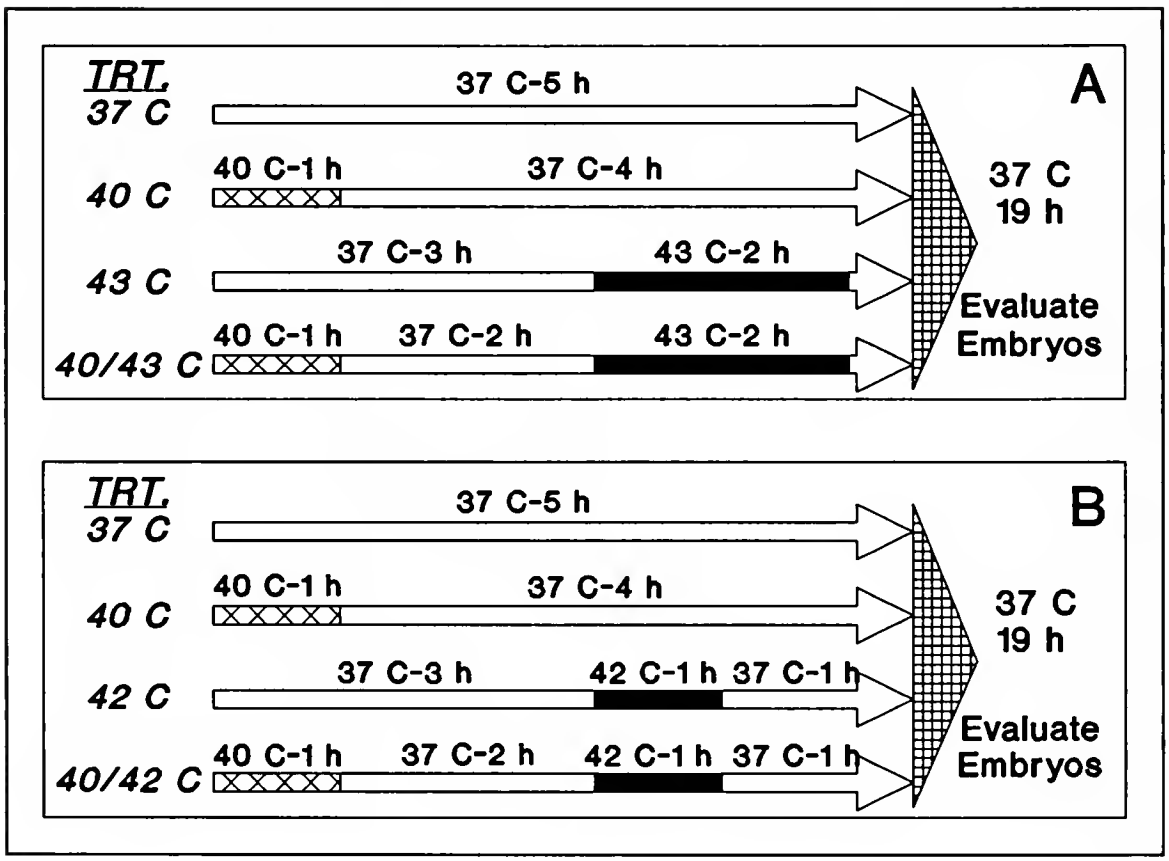

Figure 4-1. Temperature treatments used to determine the induction of thermotolerance for murine and bovine embryos. Embryos that were developed *in vitro* or *in vivo* were exposed to one of four heat treatments. **A.** Embryos were placed at: 1) 37◦C for 5 h (37◦C treatment, control); 2) 40◦C for 1 h then 37◦C for 4 h (40◦C treatment, mild heat shock); 3) 37 ◦C for 3 h then 43◦C for 2 h (43◦C heat treatment, severe heat shock); or 4) 40◦C for 1 h, 37◦C for 2 h, then 43◦C for 2 h (40/43◦C heat treatment, to observe induced thermotolerance). Embryos were then incubated (37◦C for murine embryos and 38.5◦C for bovine embryos) for 19 h and percentage of live embryos was determined. **B.** Embryos were placed at: 1) 37◦C for 4 h (37◦C treatment, control); 2) 40◦C for 1 h then 37◦C for 3 h (40◦C treatment, mild heat shock); 3) 37◦C for 3 h then 42◦C for 1 h (42◦C heat treatment, severe heat shock); or 4) 40◦C for 1 h, 37◦C for 2 h, then 42◦C for 1 h (40/42◦C heat treatment, to observe induced thermotolerance). Embryos were then incubated at 37◦C for 20 h and percentage of developing embryos was determined.

Embryonic Survival and Development

At 24 h after initiation of heat treatments (19 h following end of heat treatments), embryonic survival was determined by use of the vital stain, DAPI. Embryos were placed in DPBS (pH 7.4) containing 0.0001% DAPI at room temperature for 15 to 20 min. Embryos were washed in DPBS, placed on a microscope slide in a small volume of DPBS and evaluated for uptake of dye by nuclei of dead cells using an epifluorescent microscope with an ultraviolet excitation filter and 490 nm emission filter (Schilling *et al.*, 1979). Embryos were classified as live if less than 1/3 of cells within embryos were stained with DAPI and as dead if greater than 1/3 cells stained. Embryonic development was determined by visual observation during DAPI staining. Embryos were determined to proceed in development following heat shock if 8-cell embryos developed at least to the compact morula stage and if morulae developed to the blastocyst stage.

Statistical Analysis

Data for each stage of development were analyzed separately by ANOVA utilizing a 2 x 2 factorial design with main effects of mild (37 C versus 40 C) and severe (37 C versus 42 C or 37 C versus 43 C) heat shock and interactions (SAS, 1989). Thus, an interaction between mild and severe heat treatments was indicative of thermotolerance. Least-squares ANOVA was utilized because this procedure is considered suitable for categorical data (Wilcox *et al.*, 1990).

Results

Thermotolerance to 43 C

The induction of thermotolerance, as determined by percentage of live embryos, was dependent on stage of development, mode of embryonic development, and serum supplementation (Table 5-1). Exposure of embryos to 40 C for 1 h did not affect percentage of live embryos compared to controls at any stage, type of development, or serum supplementation. In contrast, exposure to 43 C for 2 h decreased the incidence of live embryos in all treatments (P = 0.001). At the morula stage, incubation at 40 C before exposure to 43 C (40/43 C treatment) induced thermotolerance (as indicated by mild x severe heat treatment interaction) for percentage of live embryos if embryos were developed *in vitro* (P = 0.002) but not if embryos were developed *in vivo*. Additionally, absence of serum in medium prevented the induction of thermotolerance for *in vitro* developed morulae. At the blastocyst stage, induction of thermotolerance was observed both for embryos developed *in vitro* (P = 0.02) and for embryos developed *in vivo* (P = 0.02). However, absence of serum from medium prevented induction of thermotolerance from blastocysts developed *in vitro*. Although exposure to 40 C did not affect subsequent development of embryos, 43 C for 2 h decreased development (P = 0.001) at all treatment combinations and prior exposure to 40 C did not reverse the effects of 43 C on development for any treatment combination (data not shown).

To determine whether induction of thermotolerance occurs at earlier stages of embryonic development for embryos developed *in vitro*, 8-cell embryos that were

Table 5-1. Effects of *in vitro* or *in vivo* development and presence or absence of serum on percentage of live murine embryos following heat shock.

Stage	Site of Development	Medium Supplement	Temperature Treatment	No. Replicates	Total No. Embryos	Percent Live Embryos[a]
8-cell	*in vitro*	serum	37 C	7	53	100
			40 C	7	54	96.3
			43 C	7	51	37.2
			40/43 C	7	53	58.5
Morula	*in vitro*	serum	37 C	6	25	100
			40 C	6	24	95.8
			43 C	6	26	23.1
			40/43 C	6	31	64.5
Morula	*in vivo*	serum	37 C	5	23	86.9
			40 C	5	24	95.8
			43 C	5	23	34.8
			40/43 C	5	25	32.0
Morula	*in vitro*	BSA	37 C	7	18	100
			40 C	7	20	100
			43 C	7	21	42.9
			40/43 C	7	21	47.6
Blastocyst	*in vitro*	serum	37 C	5	18	100
			40 C	5	19	100
			43 C	5	21	14.3
			40/43 C	5	18	50.0
Blastocyst	*in vivo*	serum	37 C	5	22	100
			40 C	5	26	100
			43 C	5	27	62.9
			40/43 C	5	25	92.0
Blastocyst	*in vitro*	BSA	37 C	10	20	100
			40 C	10	20	100
			43 C	10	24	0
			40/43 C	10	26	3.8

[a]43 C effect (P = 0.001) for all treatment combinations. Induced thermotolerance (determined by mild x severe heat shock interaction) was significant for *in vitro* developed embryos at 8-cell, morula and blastocyst stages and *in vivo* developed blastocysts (P = 0.02).

cultured from the 2-cell to 4-cell stage were placed in M16 medium containing 10% htFCS and exposed to heat treatments (Table 5-1). Although percentage of live embryos were not affected by exposure to 40 C compared to controls, exposure to 43 C decreased (P = 0.001) percent live embryos. Prior exposure to 40 C was successful in partially inhibiting (P = 0.02) the adverse effects of 43 C, indicating the induction of thermotolerance beginning as early as the 8-cell stage for *in vitro* developed embryos. While subsequent development of *in vitro* developed 8-cell embryos was not affected by exposure to 40 C (94.4% developed to morulae) compared to controls (100% developed), 43 C decreased (P = 0.001) subsequent development (9.8% developed) and prior incubation at 40 C was unable to reverse this effect (18.9% developed).

Thermotolerance to 42 C

A second series of experiments was performed to determine whether expression of thermotolerance capable of allowing murine embryonic development after heat shock could be observed when the heat shock used was less severe than 43 C for 2 h. An additional objective was to verify differences in the ontogeny of induced thermotolerance between embryos developed *in vitro* and *in vivo* (Table 5-2). Use of a heat shock of 42 C for 1 h in place of the more severe heat shock (43 C for 2 h) did not affect percentage of live embryos at any stage examined (data not shown) but decreased subsequent development for 8-cell embryos developed *in vitro* (P = 0.001) and for 8-cell embryos (P = 0.001) and morulae (P = 0.06) developed *in vivo*. Exposure to 40 C alone did not affect percentage of developing embryos at

Table 5-2. Ontogeny of thermotolerance for murine embryos developed *in vitro* or *in vivo* which proceeded in development following heat shock.

Stage	Site of Development	Temperature Treatment	No. Replicates	Total No. Embryos	Percent Developing Embryos[a]
8-cell	*in vitro*	37 C	4	24	95.8
		40 C	4	27	96.3
		42 C	4	29	51.7
		40/42 C	4	29	89.7
8-cell	*in vivo*	37 C	5	38	94.7
		40 C	5	42	97.6
		42 C	5	41	41.5
		40/42 C	5	41	56.1
Morula	*in vivo*	37 C	5	22	68.2
		40 C	5	24	54.2
		42 C	5	24	37.5
		40/42 C	5	24	45.8

[a]42 C effect for *in vitro* developed 8-cell embryos ($P = 0.001$), *in vivo* developed 8-cell embryos ($P = 0.001$), and *in vivo* developed morulae ($P = 0.06$). Induced thermotolerance (determined by mild x severe heat shock interaction) was significant for *in vitro* developed 8-cell embryos only ($P = 0.005$).

Table 5-3. Induction of thermotolerance in bovine blastocysts.

Temperature Treatment	Number of Replicates	Number of Embryos	Percent Live Embryos[a]
38.5 C	3	13	100
40 C	3	12	83.3
43 C	3	12	33.3
40/43 C	3	14	85.7

[a]43 C effect (P = 0.003). Induced thermotolerance (determined by mild x severe heat shock interaction) was significant (P = 0.001).

any stage or mode of development compared to controls. However, prior exposure to 40 C caused induction of thermotolerance to the 42 C heat shock for 8-cell embryos developed *in vitro* (P = 0.005), but not for 8-cell or morula stage embryos developed *in vivo*.

Thermotolerance in Bovine Blastocysts

A comparative study was conducted in bovine embryos at the blastocyst stage to determine if induction of thermotolerance can be observed during early embryonic development (Table 5-3). As with murine embryos, 40 C did not affect percentage of live embryos whereas 43 C decreased (P = 0.003) the proportion of live embryos. Prior incubation at 40 C, however, inhibited effects of 43 C on percentage of live embryos (P = 0.001).

Discussion

Induced thermotolerance is the phenomenon whereby exposure to a mild, non-lethal heat shock increases cellular resistance to a more severe, lethal heat shock. By this criterion, embryos collected at the 2-cell to 4-cell stage that underwent further development *in vitro* demonstrated induced thermotolerance beginning by at least the 8-cell stage and perhaps earlier. At this time, embryos could be made resistant to severe heat shock (43 C for 2 h or 42 C for 1 h) by prior incubation at 40 C. In contrast, embryos retrieved from oviducts or uterine horns, *i.e.* developed *in vivo*, did not display induced thermotolerance until the blastocyst stage. The ability of embryos at the blastocyst stage, developed either *in vitro* or *in vivo*, to undergo induced thermotolerance supports previous observations of induced thermotolerance

in murine blastocysts (Muller *et al.,* 1985). Differences in the ontogeny of thermotolerance between embryos developed *in vitro* and *in vivo* demonstrate that the microenvironment can alter the timing of ontogeny in preimplantation development. This effect was not an artifact of performing experiments at different times; in several instances embryos that developed *in vitro* and *in vivo* were exposed to heat treatments at the same time.

The timing of induced thermotolerance for embryos in the current experiment does not always parallel previous literature on embryonic synthesis of HSP70 proteins. Heat-inducible production of HSP68 and HSC70 did not occur for murine 8-cell embryos developed *in vitro* or *in vivo* but did occur for murine blastocysts (Morange et al., 1984; Hahnel *et al.,* 1986). Thus, murine and bovine embryos developed *in vivo* first acquired the ability to undergo induced thermotolerance at the blastocyst stage, when heat-inducible HSP70 synthesis is initiated. However, embryos cultured *in vitro* underwent induced thermotolerance before heat-stimulated production of HSP70 proteins has been reported. There are two possible explanations for this discrepancy. It is possible that the hastened ontogeny of induced thermotolerance in embryos developed *in vitro* is due to acquisition of other HSPs or biochemical mechanisms involved with stress of culture that also provides thermotolerance. It is also possible that differences in culture conditions of embryos between the present study and methods used by previous investigators might alter ontogeny of heat-inducible HSP70 synthesis. Oxidative stress occurs during mouse embryonic development in culture (Goto *et al.,* 1992) and this type of stress

stimulates HSP synthesis in culture (Omar and Pappolla, 1993). Hence, it is possible that oxidative stress during embryo culture of the present study hastened the onset of thermotolerance. The striking effect of culture on the ontogeny of thermotolerance raises concerns as to the validity of using cultured embryos to investigate timing of developmental processes, especially those related to stress.

Thermotolerance could not be induced in the absence of serum. However, constitutive and heat-inducible synthesis of HSP70 proteins occurs when *in vitro* developed embryos were cultured in medium not supplemented with serum (Morange *et al.,* 1984). Therefore, the requirement for serum components to allow thermotolerance probably involves a system other than HSP70. Serum factors that are essential for embryonic induction of thermotolerance are not defined but may include growth factors or supplemental amino acids. Maytin *et al.* (1990) reported the induction of thermotolerance in secondary keratinocytes in serum-free medium supplemented with epidermal growth factor and insulin. Serum may also supply antioxidants which aid in destroying free radical molecules produced during heat shock (Loven, 1988). The antioxidant GSH is essential for induction of thermotolerance in some cells (Mitchell *et al.,* 1983; Russo *et al.,* 1984) and may also be essential for induction of thermotolerance in murine embryos (Aréchiga *et al.,* 1992). An extracellular role of GSH has been postulated in heat-shocked bovine embryos because GSH peroxidase, an enzyme involved with scavenging free radicals, is present in serum (Avissar *et al.,* 1989), and medium supplementation with low concentrations of GSH induced embryonic resistance to heat shock (Chapter VII).

The induction of thermotolerance observed for mouse embryos is not unique for this species. It is reported here that bovine blastocysts undergo a similar induced thermotolerance response. Consequently, it is likely that early developing embryos from a variety of species may also be capable of demonstrating similar ontogeny of induced thermotolerance. Maternal hyperthermia or elevated culture temperature decreased bovine embryo development and survival during initial stages of development, whereas this effect was less evident as development progressed (Chapter III; Chapter IV). This suggests that mechanisms responsible for the induction of thermotolerance could be critical for embryonic survival in species such as cattle that are susceptible to elevated temperature effects.

Temperature treatments employed in these studies are similar to those used for previous studies of induced thermotolerance in preimplantation mouse embryos (Muller *et al.,* 1985) and postimplantation rat embryos (Mirkes, 1987). Body temperatures in mice have been reported to reach 42 C (Nowak *et al.,* 1990) but hyperthermia in most mammals is not as severe as the 43 C temperature used in some of the present studies. Although a thermotolerance response capable of preventing embryonic death to a heat treatment of 43 C for 2 h was observed, adverse effects of this heat shock on subsequent embryonic development could not be alleviated with prior incubation at 40 C for 1 h. The lack of induced thermotolerance for embryonic development at the 43 C heat treatment is probably because of the excessive severity of the heat shock. With a milder heat shock, 42 C for 1 h, pre-exposure to 40 C caused induction of thermotolerance sufficient to allow

continued development. Because induction of thermotolerance could be observed at elevated temperatures (42 C) that have been observed in mice (Nowak *et al.*, 1990), the induction of thermotolerance possibly represents a physiological mechanism used by embryos, beginning at least as early as the blastocyst stage, to prevent adverse effects of elevated temperatures or additional stresses from altering subsequent development.

CHAPTER VI
EFFECTIVENESS OF SHORT-TERM COOLING FOR ALLEVIATION OF HEAT-STRESS INDUCED INFERTILITY IN DAIRY COWS

Introduction

Pregnancy rates of lactating dairy cows are depressed during summer months in hot climates (Poston *et al.,* 1962; Stott and Williams, 1962; Rosenberg *et al.,* 1977; Badinga *et al.,* 1985; Monty and Racowsky, 1987). The peri-ovulatory oocyte and early embryo appear to be highly susceptible to heat stress. In cattle and sheep, embryonic development and viability were affected to a greater extent when heat stress occurred on the day of estrus or the day following breeding compared with heat stress on or after 3 d post-breeding (Dutt, 1963; Putney *et al.,*1989a; Chapter III). Additionally, transfer of embryos to recipients at d 7 post-estrus bypassed at least some of the deleterious effects of heat stress (Putney *et al.,* 1988c; Putney *et al.,* 1989b). Therefore, embryos become more resistant to heat stress as pregnancy progresses. Strategies which aid in alleviating maternal hyperthermia during final oocyte maturation and initial stages of embryonic development may be beneficial for enhanced fertility during periods of heat stress.

The present study investigated the efficacy of providing short-term cooling during the first few days preceding and following breeding on pregnancy rates during summer months in Florida [*i.e.,* a strategic cooling system (Hansen *et al.,* 1992)].

Previous work has indicated that short-term cooling causes either a large (Gauthier, 1983), small (Stott and Wiersma, 1976) or no (Her *et al.,* 1988) beneficial effect on pregnancy rate. In these experiments, however, possible beneficial effects may have been diminished because cows were not cooled during the peri-ovulatory period, when oocyte maturation occurs. It was hypothesized for the present study that provision of cooling from 2 to 3 d preceding breeding until 5 to 6 d following breeding would increase pregnancy rates of heat-stressed cows.

Materials and Methods

The study was completed on a commercial dairy (Larson Dairy Inc., Okeechobee, Fla) using lactating Holstein cows (first to sixth parity) greater than 50 DIM and without prior breeding during their present lactation. The study was performed during four separate periods from June to September of 1993. For each period, estrous cycles were synchronized with two intermuscular injections of 25 mg $PGF_{2\alpha}$ administered 14 d apart. Following the second $PGF_{2\alpha}$ injection, tail-heads were chalked (Paintstik; LA-CO Industries Inc., Chicago, IL) and cows were assigned randomly to treatments of either intensive cooling or shade only (control) housing. Treatments were administered for a duration of 8 d, which encompassed the period from 2 to 3 d before until 5 to 6 d following the synchronized estrus. Cows were observed for standing estrus and for removal of tail-head chalk at 0600, 1300 and 2000 h from 48 to 96 h following the second $PGF_{2\alpha}$ injection. On each occasion, cows were observed for at least 30 min while they were maintained in their respective housing. Cows observed in estrus at 0600 h were inseminated during the

evening milking (1900 h) and cows observed in estrus at 1300 h or 2000 h were inseminated during the following morning milking (0700 h) with frozen/thawed semen. All estrous observations and inseminations were performed by a single technician. Pregnancy status was verified for cows not returning to estrus by rectal palpation 90 to 120 d following breeding.

Housing systems during the treatment period were as follows. Cows exposed to short-term cooling (23 to 38 cows/period) were housed in a shaded, non-enclosed structure (9 x 25 m) which contained fans (two Turbo Aire Fans, HV22, 5570 cfm, 91 m/min flow rate at 20 m; Patterson Fan Co., Columbia, SC) and sprinklers (Senninger Irrigation Co., Orlando, FL; spacing = 2.5 m intervals; nozzle delivery = 4.26 L/min). Fans were operated continuously whereas sprinklers were operated for 3 min every 20 min as controlled by a microprocessor (Patterson Fan Co., Columbia, SC). Cows in the cooling treatment also had continuous access to a non-shaded dirt lot. For the first 3 periods of the study, controls (15 to 19 cows/period) were placed in an adjacent lot containing an aluminum-roofed shade structure (6 x 12 m) with no evaporative cooling before milking during the experimental period. For the fourth period, controls (47 cows) were placed with additional cattle not on the experiment in a nearby lot which contained several portable shade structures using shade-cloth (80% shade cover; Donovan Enterprises, Stuart, FL) and received evaporative cooling for approximately 30 min before each milking. Cows were maintained in their respective housing treatments at all times, except for milking (2X, 0700 to 0730 h and 1900 to 1930 h). At times other than the experimental period, cows were

maintained with the lactating herd in large lots with portable shade cloths and access to sprinklers for 30 min prior to milkings.

During each period, dry bulb temperature was recorded at 0800, 1200 and 1600 h daily. Additionally, black bulb temperature, a measurement of heat exchange that considers dry bulb temperature, solar radiation and wind speed, was recorded within cooling and shade structures and in a non-shaded area in close proximity to treatment structures at 0800 h, 1200 h and 1600 h daily.

Reproductive data were analyzed by least squares ANOVA [GLM, (SAS, 1989)]. Data were first analyzed using a complete model consisting of all main effects and interactions. Subsequently, data were reanalyzed using a reduced model which consisted of main effects and significant interactions. Categorical data in which significant effects were found by ANOVA were also analyzed by CATMOD (SAS, 1989) using the reduced models developed for ANOVA. Percentage of cows observed in estrus, percentage of cows in standing estrus, time of estrous observation (AM or PM) and time from $PGF_{2\alpha}$ injection to first estrous detection were analyzed using a model that included main effects of treatment (short-term cooling or shade only), parity (first, second, or third and greater lactation) and period (first through forth period). The percentage of bred cows that were pregnant were analyzed for main effects of treatment, parity, period, type of estrous observation (standing or chalk removal only) and time of breeding (AM versus PM). Parity effects were partitioned using orthogonal comparisons of first versus second parity, and first and second versus third and greater parity.

Results

As shown in Table 6-1, the cooling system was effective in reducing heat stress. Black globe temperatures were 3.1 to 5.0 C lower in the cooling facility than in the shade area.

The proportion of cows observed in estrus after synchronization did not differ significantly between treatments, although the proportion of cows detected in estrus tended to be greater for cooled cows (Table 6-2). Provision of cooling did not alter the proportion of estrous cows observed in standing estrus (versus detection by tail-head chalk removal only), time of day when cows were first observed in estrus, or the duration from the second $PGF_{2\alpha}$ injection to first observation of estrus. Pregnancy rate was increased from 6.2% for cows placed in the shade treatment to 16.0% for cows housed in the cooling treatment. This effect was significant as determined by least squares ANOVA (P = 0.02) but not by CATMOD. Proportion of pregnant cows was not affected by parity, type of estrous observation, or time of breeding (AM versus PM) or any interactions.

Parity affected estrous observations. Fewer (P = 0.05) primiparous cows were observed in estrus (35.1%; n = 94 cows) than cows in their second (51.1%; n = 47 cows) or third and greater (49.0%; n = 51 cows) lactations. Additionally, the proportion of estrous cows observed in standing estrus was lower (P = 0.02) for cows in their first (57.6%; n = 33) and second (50.0%; n = 24) lactations than cows in their third or greater lactation (72.0%; n = 25). No effects of parity were observed

Table 6-1. Environmental temperatures for cooling and shade treatments.

Time	Black Bulb Temp.(C)			Dry Bulb Temp. (C)
(h)	Cooling	Shade	Non-Shade	
0800	23.8 ± 0.4	26.9 ± 0.6	29.2 ± 0.8	25.4 ± 0.6
1200	28.9 ± 0.4	33.3 ± 0.5	39.9 ± 0.8	33.7 ± 0.6
1600	28.4 ± 0.4	33.4 ± 0.5	37.9 ± 0.8	33.9 ± 0.6

Table 6-2. Reproductive responses of cows exposed to cooling and shade treatments from 2 to 3 d before until 5 to 6 d following breeding.

Response	Cooling	Shade
Number of Cows	106	86
DIM[a]	74.1 ± 3.9	79.4 ± 5.3
Number Observed in Estrus	50/106 (46.2%)	32/86 (37.2%)
Number Standing	31/50 (62.0%)	18/32 (56.2%)
Number Chalk Removed	19/50 (38.0%)	14/32 (43.8%)
First Estrus Observation		
AM	26/50 (52.0%)	21/32 (65.6%)
PM	24/50 (48.0%)	11/32 (34.4%)
h post-$PGF_{2\alpha}$ to first estrus observation[a]	63.3 ± 3.4	56.8 ± 4.5
Number Pregnant[b]	8/50 (16.0%)	2/32 (6.2%)

[a]Represent least-squares means and SEM.

[b]Treatment effect (P = 0.02, ANOVA; Not Significant, CATMOD).

for the time of estrous observation and interval from $PGF_{2\alpha}$ injection to estrus. Additionally, no treatment x parity interactions were observed for any responses.

Discussion

Cooling cows from 2 to 3 d before until 5 to 6 d following breeding was successful in improving pregnancy rates during summer months in Florida. However, this short-term cooling regimen was not able to restore pregnancy rates to levels comparable to those occurring in the winter (32.5%) at the same farm. Another experiment in which cows were cooled for the first 4 d following insemination also reported a slight increase in pregnancy rates (Stott and Wiersma, 1976). In another experiment (Her *et al.,* 1988), there was no effect of cooling but the degree of hyperthermia for control cows was slight. Gauthier (1983), in contrast, found a large increase in pregnancy rate with cooling during the first 10 d of pregnancy (13% in controls to 53% in short-term cooled cows) but few cows were used. It was anticipated that the cooling system used in the present experiment would be more successful than previous experiments because cooling was initiated before estrus, when final follicular development and oocyte maturation occurs. Failure to achieve optimal pregnancy rates implies that heat stress before or after the cooling period had detrimental effects on embryonic development. One possibility for this marginal effects is that hyperthermia may have occurred in cooled cows. This question cannot be addressed, however, because rectal temperatures were not measured in the present study. Heat stress from d 8 to 16 of pregnancy had slight detrimental effects on embryonic development (Biggers *et al.,* 1987) and increased $PGF_{2\alpha}$ secretion from

the uterus (Putney *et al.,* 1989c; Wolfenson *et al.,* 1993). Additionally, deleterious influences of heat stress on follicle dynamics have also been implicated with causing decreased fertility in cattle (Badinga *et al.,* 1993).

The proportion of cows detected in estrus was not improved by short-term cooling, even though several studies indicate that heat stress reduces the intensity and duration of estrus (Gangwar *et al.,* 1965; Roller and Stombaugh, 1974; Wolff and Monty, 1974; De Silva *et al.,* 1981; Gwasdauskas *et al.,* 1981; Thatcher and Collier, 1986; Wolfenson *et al.,* 1988). The lack of differences in estrous detection in experiment was not due to use of an estrous detection aid since no differences in incidence of cows observed in standing estrus versus those detected by tail-head chalk removal only were evident between treatments. The percentage of cows observed in estrus was low, signifying that either the efficiency of estrous detection was low for both groups or that many cows had not yet resumed estrous cycles and were incapable of responding to $PGF_{2\alpha}$ treatment. Interestingly, the fertility of cows inseminated by observation of tail-head chalk removal only was the same as that for cows inseminated based on observing standing estrus, suggesting that tail-head chalk is an appropriate means of detecting estrus in cows subjected to estrous synchronization regimens.

In conclusion, cooling cows from 2 to 3 d before until 5 to 6 d after breeding improved pregnancy rates in heat-stressed cows but the magnitude of response was not large. The marginal success of the strategic cooling system does not make it a practical system for on-farm use as presently constructed.

CHAPTER VII
THERMOPROTECTION OF BOVINE AND MURINE EMBRYOS FROM HEAT SHOCK BY GLUTATHIONE AND TAURINE

Introduction

Many aspects of cellular function are disrupted by exposure of mammalian cells to temperatures associated with hyperthermia. Mammalian embryos are particularly sensitive to heat shock; culture of embryos at elevated temperatures causes retardation in development, malformations and embryonic death (Ulberg and Sheean 1973; Mirkes, 1987; Arechiga *et al.,* 1992; Malayer *et al.,* 1992; Chapter IV; Chapter V). Hypersensitivity of oocytes and early embryos to heat shock is likely due to their inability to produce HSPs or other intracellular molecules in response to heat shock. However, even at the blastocyst stage in mice, when HSPs are synthesized in response to elevated temperatures (Wittig *et al.,* 1983), heat shock still decreases embryonic development and survival (Chapter V).

Free radical production has been proposed as one mechanism by which heat shock alters cellular development and survival. Loven (1988) proposed that generation of $\cdot O_2^-$, H_2O_2, and $\cdot OH$ is a major cause of heat-shock induced cell death through actions such as peroxidation of lipid membranes and damage to proteins. Intracellular antioxidants such as GSH and enzymes involved in removal of free radicals increase upon exposure to heat in thermotolerant cells but not

thermosensitive cells (Omar *et al.,* 1987; Russo *et al.,* 1984). Likewise, intracellular injections of GSH induced thermotolerance in cells (Lumpkin *et al.,* 1988), whereas depletion of cellular GSH reduced cellular resistance to heat shock (Russo *et al.,* 1984). It may be possible to increase embryonic resistance to heat shock, therefore, by extracellular provision of antioxidants. Work with morula-stage murine embryos indicates that thermal resistance can be increased by medium supplementation with taurine (Malayer *et al.,* 1992), alanine (Malayer *et al.,* 1992) and vitamin E (Aréchiga *et al.,* 1994a). Identification of antioxidants that block effects of heat shock on embryos at stages sensitive to maternal hyperthermia could lead to the use of these molecules under field conditions to improve fertility during heat stress.

Two antioxidants, GSH and taurine, are of particular interest as embryonic thermoprotectants. Glutathione is known to play an important role in intracellular thermoprotection (Lumpkin *et al.,* 1988; Russo *et al.,* 1984). Aréchiga *et al.* (1992) reported that intracellular depletion of GSH from murine embryos increased thermal sensitivity, whereas increased intracellular GSH levels increased thermal resistance. No reports exist of thermoprotective modulation by GSH when provided extracellularly; however, extracellular GSH can be oxidized by $\cdot O_2^-$ and H_2O_2 from neutrophils (Thomas *et al.,* 1988) and extracellular sources of GSH peroxidase exist in blood (Avissar *et al.,* 1989). Taurine is present in millimolar concentrations within the oviduct and uterus of mammals (Fahning *et al.,* 1967; Van der Horst and Brand, 1969; Casslén, 1987) and has been shown to protect bovine lymphocytes and mouse embryos from heat shock at similar concentrations (Ulberg and Sheean, 1973).

Therefore, studies were conducted to determine whether extracellular administration of GSH and taurine increased resistance of bovine and murine embryos to adverse effects of heat shock.

Materials and Methods

Experiment 1

Seven groups of nonlactating dairy and beef cows were superovulated over a 4 mo period during winter mo. Estrous cycles were synchronized by two injections of $PGF_{2\alpha}$ (25 mg) given at 11 d intervals. Injections of 6, 5, 4 and 3 mg FSH-P were administered twice on d 10, 11, 12 and 13 postestrus, respectively (estrus = d 0). On d 12, $PGF_{2\alpha}$ (25 mg) was administered twice following FSH-P injection. On the evening of d 13, cows were placed with a fertile bull and were artificially inseminated 8, 18 and 28 h after onset of standing estrus. If estrus was not observed by the morning of d 14, cows were injected with 3 mg FSH-P and placed back with bulls until standing estrus was observed. Embryos were recovered 6 d after estrus from 40 cows by nonsurgical flushing of the uterus (Drost, 1986) or by flushing excised reproductive tracts after slaughter.

Only morulae with no or few degenerative cells were used in the experiment. Groups of 4 to 6 morulae were placed in 0.5 ml modified Hams-F10 medium (containing 10% (v/v) htFCS, 0.3 mg/ml L-glutamine, 100 U/ml Na penicillin-G and 100 μg/ml streptomycin sulfate) supplemented with 50 nM GSH (Sigma Chemical Co.), 50 mM taurine (Sigma Chemical Co.) or neither. Embryos were incubated in a humid environment (5% CO_2/ 95% air) at 38.5 C for 2 h before being incubated

at 42.0 C or 38.5 C for 2 h. Embryos were then incubated at 38.5 C for 20 h. After 24 h, embryos were removed from medium and stained with DAPI. After 20 min incubation at room temperature, dead cells within embryos emit fluorescence whereas live cells do not (Schilling et al., 1979). Embryos were classified as live if less than one-third of cells within the embryo stained positive for DAPI and were classified as dead if greater than one-third stained positive. Embryos were also classified according to whether development proceeded from the morula to blastocyst stage (presence of blastocele cavity).

Experiment 2

Murine 2-cell embryos were retrieved from superovulated ICR females as in Chapter IV and described in Appendix B. Embryos were placed in groups of 5 to 10 in 5 to 7 μl drops of M16 medium containing 0.4% BSA covered with double-extracted silicon oil and incubated in an atmosphere of 5% CO_2 in humid air until the morula stage. Morulae were then transferred in groups of 5 to 10 to microdrops of M16 medium containing 10% htFCS and 0, 50 nM or 5 μM GSH. After 2 h at 37 C, embryos were incubated at either 37 or 43 C for 2 h. Embryos were then cultured for an additional 20 h at 37 C before evaluations of viability (DAPI Stain) and development to the blastocyst stage.

Experiment 3

To verify results of experiment 2, murine morulae developed in culture were placed in groups of 5 to 10 in microdrops of M16 medium containing 10% htFCS and 0 or 5 μM GSH. After 2 h at 37 C, embryos were incubated at either 37 or 43 C for

2 h. Embryos were then cultured for an additional 20 h at 37 C before evaluation of viability (DAPI Stain) and development to the blastocyst stage.

Experiment 4

The procedures used to produce *in vitro* derived embryos were modifications of existing procedures (Parrish *et al.,* 1988; Xu *et al.,* 1992; Hernandez-Ledezma *et al.,* 1993) as outlined in Appendix A. Cleavage rate averaged 67.4% and rate of parthenogenesis averaged 5.6% during experiment 4 and 5.

Bovine 2-cell embryos (d 2 post-IVF) were transferred to 600 μl modified CZB medium containing 50 nM GSH, 50 mM taurine or no antioxidant supplementation. Cultures were equilibrated for 1 h at 39 C before exposure to 41 C for 3 h. Embryos were then maintained in CZB medium containing antioxidants at 39 C until d 3 post-IVF, when embryos were transferred to modified TCM-199 medium without additional antioxidants. Development was assessed on d 5 post-IVF.

Experiment 5

The monoethyl ester of GSH was synthesized using the sulfuric acid esterification procedure described by Anderson and Meister (1989). Identity of the GSH ester was confirmed to be of >90% purity by thin layer chromatography using MK6F plates (Whatman, Hillsboro, OR), solvent (n-propanol:acetic acid:water, 16:3:5, v/v) and ninhydrin (Whatman, Hillsboro, OR) as described by Anderson and Meister (1989).

Bovine embryos at the 2-cell stage (d 2 post-IVF) were transferred to 600 μl modified CZB medium containing 0, 10 μM, 100 μM, 1 mM or 10 mM GSH ester.

Cultures were equilibrated for 3 h at 39 C before exposure to 41 C for 3 h. Embryos were then maintained in CZB medium containing antioxidants at 39 C until d 3 post-IVF, when embryos were transferred to modified TCM-199 medium without additional antioxidants. Development was assessed on d 5 post-IVF.

Statistical Analysis

Experiment 1 was analyzed using ANOVA (SAS, 1989) in three ways. A 2 x 3 factorial design was used to determine heat shock effects and overall treatment differences. Additionally, data were analyzed in 2 x 2 factorial designs to separately evaluate effects of GSH and taurine. Variables in all analyses included effects of temperature, antioxidant and temperature x antioxidant interaction. Experiment 2 and 3 were analyzed as a 2 x 3 and a 2 x 2 factorial design, respectively, using ANOVA for main effects of temperature, GSH (or dose of GSH) and temperature x GSH interaction.

For experiment 4 and 5, each experiment was performed on several different days, using one or more wells or microdrops (replicates) of embryos per treatment on each day. Treatment effects on the number of embryos undergoing development in each replicate (*i.e.*, each well or microdrop) were analyzed by ANOVA. Class effects in the model were temperature, day, antioxidant (or dose of GSH ester) and interactions. For both experiments, the total number of embryos in each replicate were used as a covariate in these analyses. Experiment 4 was analyzed in three ways; in a 2 x 3 factorial design (complete data set) or in 2 x 2 factorial designs to separately evaluate effects of GSH and taurine. Probability values represent results

from these analyses. To calculate least squares means for percentage of embryos undergoing development, percent development for each replicate were also analyzed by ANOVA. Standard errors represent pooled standard errors since variance was not heterogenous in either experiment.

Results

In experiment 1 (Table 7-1), incubation of bovine morulae at 42.0 C for 2 h decreased percent live embryos (P = 0.001) and percent development to blastocysts (P = 0.001). At 42.0 C, but not at 38.5 C, GSH increased embryo viability (P = 0.02) and development (P = 0.05). However, GSH only partially blocked the effects of heat shock. Taurine had no effect on bovine embryos cultured at 38.5 C. At 42.0 C, taurine increased embryo viability (P = 0.02) but did not affect development. The beneficial effect of taurine was of smaller magnitude than that for GSH.

For experiment 2 (Table 7-2), exposure of murine morulae to 43 C for 2 h decreased percentage of live embryos (P = 0.001) and percentage of embryos developing to blastocysts (P = 0.001). GSH reduced the effects of 43 C on murine embryo viability (P = 0.05) but not development. At 37 C, 50 nM and 5 μM GSH increased (P = 0.001) development of murine morulae to blastocysts. An additional study (experiment 3; Table 7-3) was conducted to verify beneficial effects of GSH on viability of heat-shocked murine embryos. 5 μM GSH reduced the effect of 43 C on percent live embryos (P = 0.001) but did not affect percent development to the blastocyst stage. In this experiment, GSH was unable to improve development at 37 C.

Table 7-1. Effect of glutathione and taurine on viability and development of bovine morulae to 42 C heat shock for 2 h.

Treatment	Number of Replicates	Number of Embryos	Percent Live Embryos[a]	Percent Developing to Blastocysts[b]
38.5 C				
control	6	20	95.0	80.0
50 nM GSH	6	20	95.0	80.0
50 mM taurine	6	20	90.0	75.0
42.0 C				
control	6	23	26.1	30.4
50 nM GSH	6	22	72.0	54.5
50 mM taurine	6	22	50.0	31.8

[a] Effect of heat shock (P = 0.001), heat shock x GSH (P = 0.02) and heat shock x taurine (P = 0.02).

[b] Effect of heat shock (P = 0.001) and heat shock x GSH (P = 0.05).

Table 7-2. Dose-dependent effect of GSH on viability and development of murine morulae exposed to a heat shock of 43 C for 2 h.

Treatment	Number of Replicates	Number of Embryos	Percent Live Embryos[a]	Percent Developing to Blastocysts[b]
37 C				
0	4	41	100	59
50 nM GSH	4	42	100	93
5 μM GSH	4	40	100	90
43 C				
0	4	43	40	0
50 nM GSH	4	51	49	0
5 μM GSH	4	48	58	0

[a]Effect of heat shock ($P = 0.001$), GSH ($P = 0.05$) and heat shock x GSH ($P = 0.05$).

[b]Effect of heat shock, GSH and heat shock x GSH ($P = 0.001$).

Table 7-3. Effect of GSH on viability and development of murine morulae exposed to a heat shock of 43 C for 2 h.

Treatment	Number of Replicates	Number of Embryos	Percent Live Embryos[a]	Percent Developed to Blastocysts[b]
37 C				
0	4	33	100	64
5 μM GSH	4	33	97	58
43 C				
0	4	29	38	10
5 μM GSH	4	40	78	15

[a]Effect of heat shock, GSH and heat shock x GSH ($P = 0.001$).

[b]Effect of heat shock ($P = 0.01$) and heat shock x GSH ($P = 0.05$).

Two experiments (experiment 4 and 5) were performed to determine if administration of selective antioxidants protected 2-cell bovine embryos from heat shock. In experiment 4, effects of 50 nM GSH and 50 mM taurine were evaluated (Table 7-4). Heat shock of 41 C for 3 h decreased (P = 0.06) subsequent embryonic development. Although effects of antioxidant treatment (P = 0.02) and heat shock x antioxidant treatment (P = 0.02) were observed, there were no thermoprotective effects of GSH or taurine. Indeed, taurine caused decreased development of embryos at 39 C. For experiment 5, administration of a membrane permeable form of GSH, GSH ester, was examined for ability to prevent effects of heat shock at doses of 10 μM, 100 μM, 1 mM and 10 mM (Figure 7-1). At the highest concentration (10 mM), GSH ester totally inhibited subsequent development at 39 C (0/38 developed) and 41 C for 3 h (0/26 developed). Therefore, this treatment was removed from subsequent analysis. Heat shock of 41 C for 3 h decreased embryonic development (P = 0.002) and there was a heat shock x GSH ester interaction (P = 0.04). However, this interaction represents decreased development of control embryos administered 100 μM GSH ester. Therefore, GSH ester was unable to prevent heat shock effects on embryonic development at any concentration examined.

Discussion

Present experiments represent the first report of extracellular GSH administration that enhances embryo survival in response to heat shock. Furthermore, the beneficial effects of GSH were demonstrated in a system highly

Table 7-4. Effect of glutathione and taurine on subsequent development of 2-cell bovine embryos exposed to 41 C heat shock for 3 h.

Treatment	Number of Replicates	Number of Embryos	Percent Embryos Developing to ≥16-cells*
39 C			
control	6	99	46.3 ± 3.7
50 nM GSH	5	80	40.9 ± 4.1
50 mM taurine	4	58	13.8 ± 4.6
41 C			
control	6	97	24.6 ± 3.7
50 nM GSH	6	98	30.0 ± 3.7
50 mM taurine	4	61	26.6 ± 4.6

*Data represent least-squares means ± SEM of embryonic development on d 5 post-IVF. Overall, subsequent embryonic development was affected by heat shock (P = 0.06), antioxidant treatment (P = 0.02) and heat shock x antioxidant treatment (P = 0.02). When only GSH and control treatments were analyzed, there was an effect of heat shock (P = 0.006) but GSH treatment or heat shock x GSH treatment effects were not significant. When taurine and control treatments were analyzed, there were effects of taurine treatment (P = 0.01) and heat shock x taurine treatment (P = 0.006).

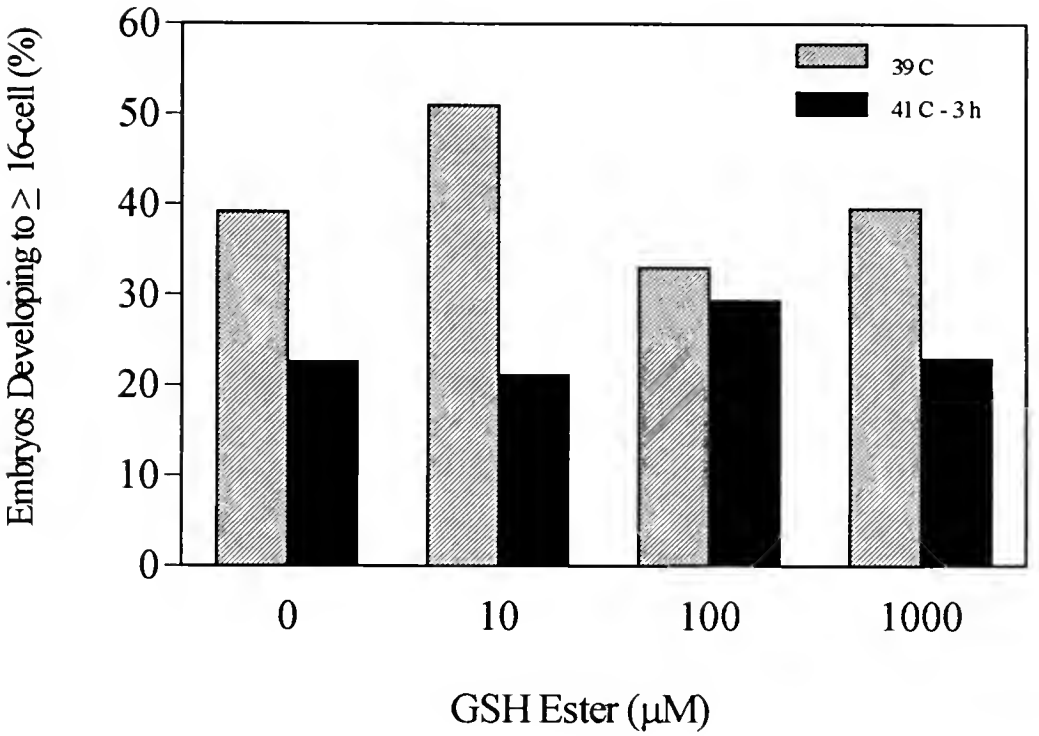

Figure 7-1. Absence of beneficial effects of GSH ester for alleviating heat shock induced decreases in subsequent development of 2-cell bovine embryos. Data represent least-squares means of 54 to 78 embryos in 3 to 4 replicates with a pooled SEM = 6.5%. Although heat shock decreased embryonic development (P = 0.002), there was no effect of GSH ester but there was a GSH ester x heat shock interaction (P = 0.04) on subsequent development.

sensitive to heat shock. While the importance of intracellular GSH in protecting cells from heat shock is well established (Russo *et al.,* 1984; Omar *et al.,* 1987; Lumpkin *et al.,* 1988; Aréchiga *et al.,* 1992), this experiment infers an additional, extracellular role for GSH in protecting embryos. Since intracellular concentrations of GSH are 200 to 200,000 fold higher than were supplemented in medium (Kosower, 1976), it is unlikely that GSH supplementation in the medium significantly altered antioxidant status within the cell. Rather, GSH probably prevented lipid peroxidation of embryonic membranes from extracellular reduced oxygen species by serving directly as an antioxidant (Thomas *et al.,* 1988) or as a substrate for extracellular GSH peroxidase (Avissar *et al.,* 1989).

Medium supplementation with taurine protected heat-shocked bovine embryos at the morula stage, but beneficial effects were minimal. A similar, slightly beneficial effect of taurine has been reported for heat-shocked mouse embryos (Malayer *et al.,* 1992). Taurine is probably also acting as an antioxidant (Wright *et al.,* 1986; Green *et al.,* 1991). Interestingly, millimolar concentrations of taurine are present in the uterus and oviduct (Fahning *et al.,* 1967; Van der Horst and Brand, 1969; Casslén, 1987), suggesting taurine may play a physiological role in regulating embryonic function, either as a thermoprotectant or through other mechanisms.

In contrast to bovine morulae, deleterious effects of heat shock on subsequent development was not reduced with GSH administration in murine morulae. This was probably caused by the severity of 43 C heat shock treatment on murine embryos. In Chapter IV, induction of thermotolerance reversed effects of 43 C on viability but

not development. If the severity of heat shock was reduced (42 C for 1 h), however, induction of thermotolerance reduced effects of heat shock on development. Therefore, it is possible that GSH would also reverse effects of heat shock on embryonic development if the severity of heat shock was reduced.

In contrast to bovine and murine embryos at the morula stage, none of the antioxidants tested were successful in protecting 2-cell embryos from adverse effects of heat shock. It is possible that this difference in response is due to differences in stage of embryonic development. Antioxidants may not be effective at the 2-cell stage if these embryos have not developed mechanisms which confer thermal resistance that are necessary for eliciting thermoprotective effects of antioxidants. Membrane transport systems for taurine (Wright *et al.,* 1986) and GSH (Meister and Anderson, 1983), or interdependent antioxidant systems (*i.e.,* GSH peroxidase) if acting intracellularly, likewise may not be present at stages of early development in embryos. Lack of thermoprotection by administration of the membrane-permeable GSH ester indicates that the ineffectiveness of GSH in preventing effects of heat shock was not caused by failure of entry into the cell. It is also possible that concentrations used or duration of embryo exposure to antioxidants prior to heat shock were not adequate in enhancing antioxidant status of embryos. Finally, it must be considered that heat shock involves cellular damage independent of free radicals. For example, temperature-dependent changes in protein and membrane structure may be sufficient to prevent development in early embryos (Lepock *et al.,* 1983; Konings, 1988). Even in mouse morulae, antioxidants were more effective in

preventing effects of heat shock on percentage of live embryos (determined by live/dead stain) than on subsequent stages of development (Malayer *et al.,* 1992; Aréchiga *et al.,* 1994a; present experiment).

Previous studies also have observed beneficial effects of taurine (Dumoulin *et al.,* 1992) and GSH (Legge and Sellens, 1991) on murine embryo development at 37 C. The general lack of beneficial effects of antioxidants at 39 C in the present studies can be attributed to the short duration of antioxidant treatment. High concentrations of GSH ester adversely affected development of 2-cell bovine embryos, which probably was due to an observed increase in the acidity of medium. Additionally, 50 mM taurine inhibited development of 2-cell embryos at 39 C, even though similar concentrations are present in uterine and oviductal fluids (Fahning *et al.,* 1967; Van der Horst and Brand, 1969; Casslén, 1987). Perhaps, toxicity of taurine was caused by increased osmolarity of medium or another unidentified type of cellular stress. In contrast to the toxic effect of taurine at 39 C, no toxic effect was apparent with exposure of 2-cell embryos to 41 C for 3 h, perhaps because the thermoprotective effect of taurine compensated for any toxic effects. Perhaps, lower concentrations of taurine may be effective in protecting early stage bovine embryos from heat shock.

In conclusion, GSH and taurine were successful in reducing effects of heat shock on bovine and murine embryo viability at the morula stage. However, none of the antioxidants examined were successful in reducing heat shock effects for 2-cell bovine embryos. Therefore, further research on identification of thermoprotective

molecules should be directed at protection during earlier stages of embryonic development, when embryos are highly sensitive to effects of elevated temperature.

CHAPTER VIII
GENERAL DISCUSSION

The two major conclusions from this dissertation were that bovine embryos gain resistance to elevated temperatures as development progresses and antioxidants can serve as thermoprotectant molecules for cultured mammalian embryos at certain stages of development.

In the first experiment (Chapter III), embryos gained resistance to effects of maternal heat stress as development progressed (Figure 8-1). Development and viability of embryos at d 8 of pregnancy was decreased by maternal heat stress on d 1 of pregnancy but was not affected by heat stress on or after d 3 of pregnancy. This pattern of ontogeny is similar to that for ovine embryos (Dutt, 1963). Present findings do not imply that bovine embryos are completely resistant to effects of maternal heat stress by d 3 of pregnancy; heat stress of greater severity or duration than that used in this study could possibly decrease embryonic survival before or after d 1. Nonetheless, results from this experiment and a previous report on susceptibility of oocytes to heat stress during final maturation (Putney *et al.*, 1989a) make it possible to identify a period of oocyte and embryonic development when heat stress effects are highly detrimental to pregnancy rates.

Developmental acquisition of embryonic resistance to elevated temperatures also was observed in cultured bovine embryos (Chapter IV; see Figure 8-1). *In vitro*

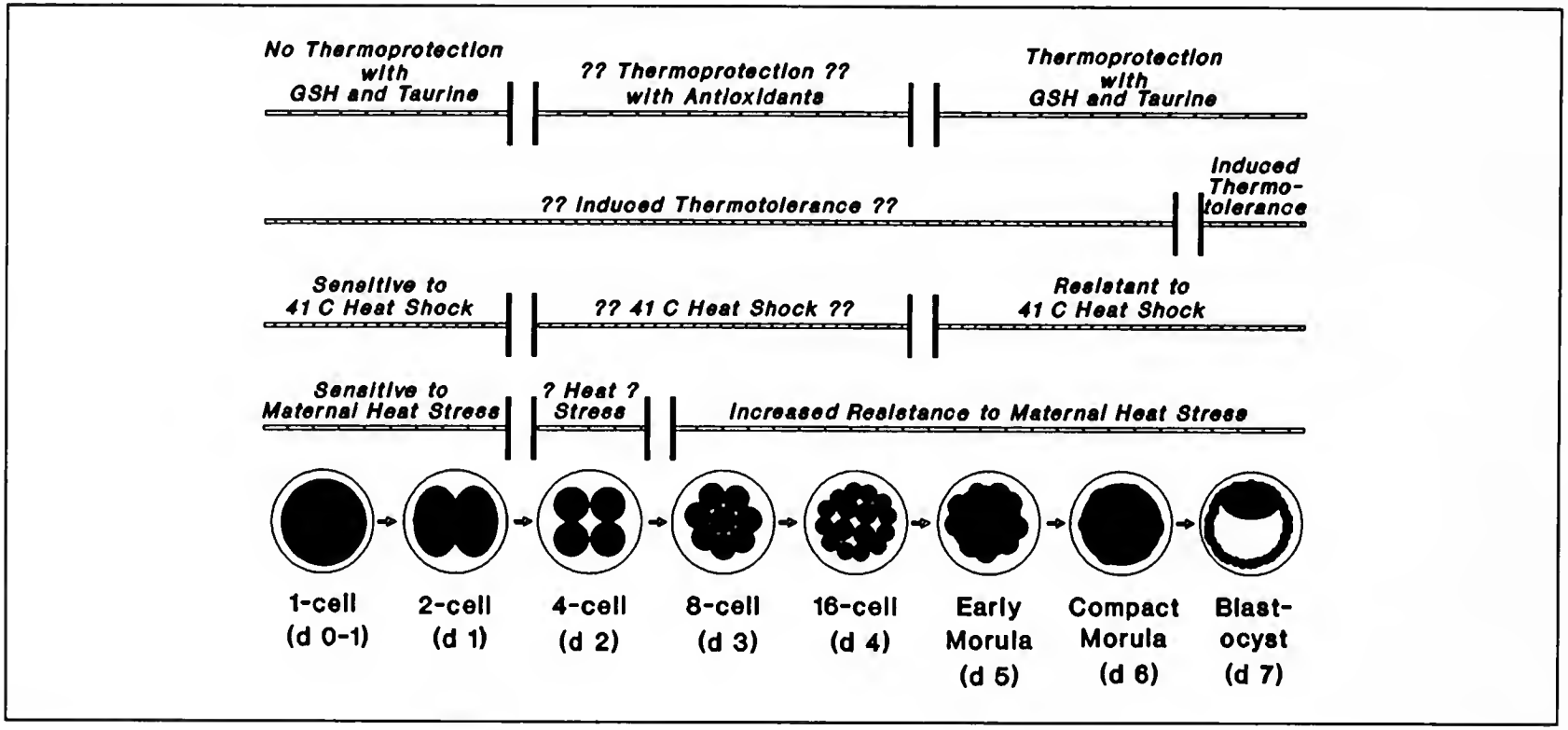

Figure 8-1. Current model depicting developmental changes in resistance of bovine embryos to elevated temperatures. Bovine embryos become resistant to effects of elevated temperature as development progresses: Embryos at d 1 of pregnancy are more sensitive to maternal heat stress effects than embryos on or after d 3 of pregnancy (Chapter II). Additionally, function of cultured 2-cell bovine embryos is decreased by exposure to 41 C for 3 h whereas morulae are not affected (Chapter III). The efficacy for use of antioxidants is also dependent upon stage of development. At the morula stage GSH and taurine are capable of reducing heat stress effects on embryonic viability and development (Chapter VI). However, effects of heat shock could not be reduced by GSH or taurine at the 2-cell stage (Chapter VI).

development of 2-cell bovine embryos was reduced with exposure to 41 C for 3 h, whereas development of morula-stage embryos was not affected by this heat shock. This study also suggests that elevated temperature directly alters embryonic development because the heat shock used was similar to body temperatures achieved in heat-stressed cattle (Putney *et al.,* 1989; Chapter IV). Hence, the gain in embryonic resistance to elevated temperatures during development probably involves acquisition of intracellular mechanisms which confer thermal resistance.

One way to determine the presence of these intracellular mechanisms is through characterizing the onset of the induced thermotolerance response. Bovine blastocysts were capable of undergoing induced thermotolerance (Chapter V), so that, by the blastocyst stage, bovine embryos contain intracellular biochemical systems which confer thermal resistance. Murine embryos were used to further characterize the ontogeny of this response (Chapter V; see Figure 8-2). For embryos developed *in vitro*, induced thermotolerance was first observed at the 8-cell stage whereas embryos developed *in vivo* did not display induced thermotolerance until the blastocyst stage. The mechanism by which thermotolerance is acquired may not include heat-induced production of HSP70. Embryos developed *in vitro* (Morange *et al.,* 1984) and *in vivo* (Wittig *et al.,* 1983; Hahnel *et al.,* 1986) did not synthesize HSP70 in response to heat at the 8-cell stage but did at the blastocyst stage. One possibility is that present culture conditions altered the heat-induced synthesis of HSP70 during *in vitro* development. Another possibility, however, is that the hastened occurrence of induced thermotolerance for in embryos developed *in vitro*

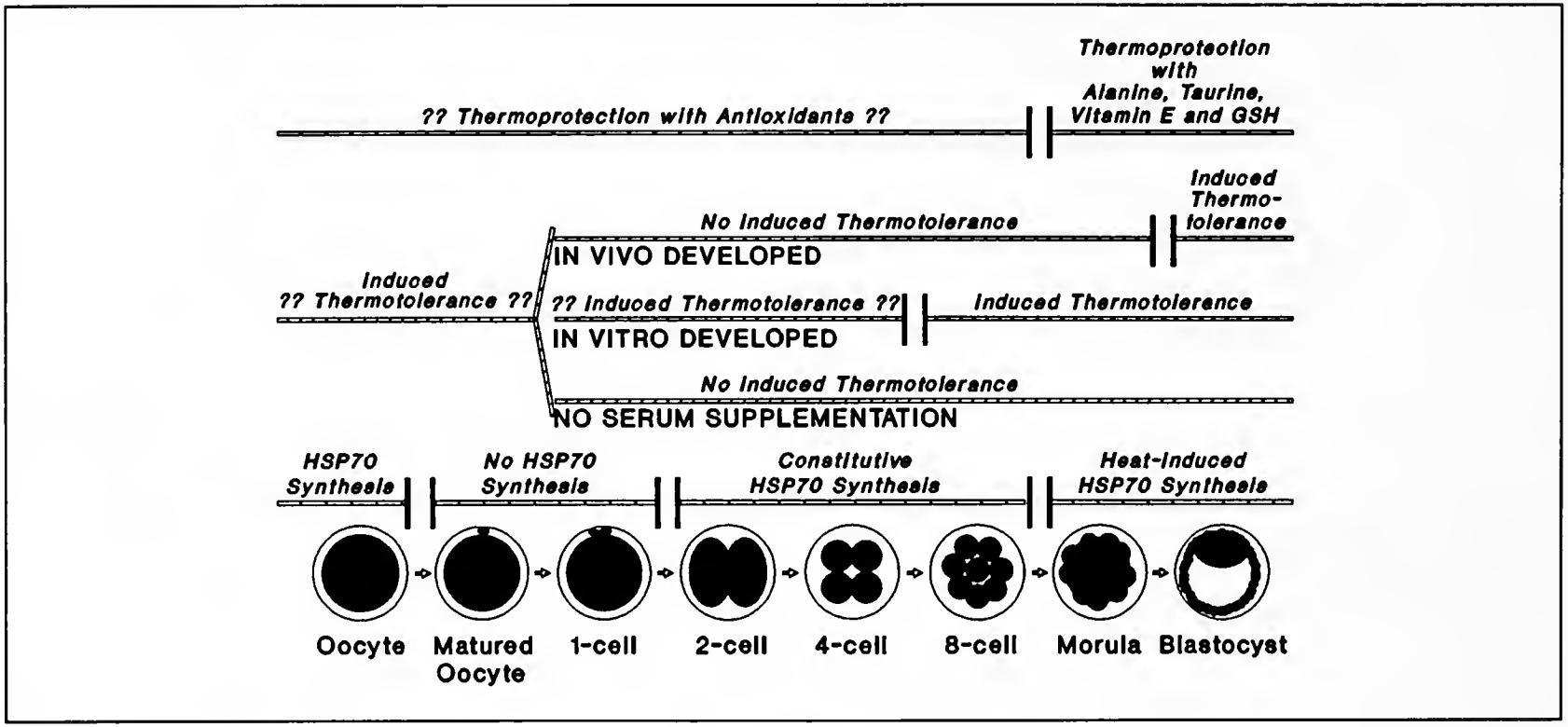

Figure 8-2. Current model of events associated with the ontogeny of thermal resistance in murine embryos. As development progresses, murine embryos gain the ability to undergo induced thermotolerance. For embryos developed *in vivo*, thermotolerance is induced beginning at the blastocyst stage, whereas induced thermotolerance occurrs at the 8-cell stage for embryos developed *in vitro* (Muller *et al.*, 1985; Chapter IV). However, serum supplementation is required for induction of thermotolerance (Chapter IV). The ontogeny of thermotolerance is not associated with previous reports on the ontogeny of HSP70 synthesis (Wittig *et al.*, 1983; Morange *et al.*, 1984; Muller *et al.*, 1985; Hahnel *et al.*, 1986). Effects of heat shock is reduced by supplementation of GSH (Chapter VI), alanine (Malayer *et al.*, 1992), taurine (Malayer *et al.*, 1992) and vitamin E (Aréchiga *et al.*, 1994).

is due to acquisition of other HSPs (Lindquist and Craig, 1988; Burel *et al.,* 1992) or other biochemical mechanisms involved with thermotolerance. This observation also raises concerns to the validity of using cultured embryos to investigate timing of developmental processes, especially those related to stress.

Thermotolerance of murine embryos could not be induced in the absence of serum (Chapter V; see Figure 8-2). However, constitutive and heat-inducible synthesis of HSP70 proteins occurs when embryos are cultured in medium not supplemented with serum (Bensaude *et al.,* 1983; Wittig *et al.,* 1983; Morange *et al.,* 1984; Hahnel *et al.,* 1986). Therefore, the essential role of serum for thermotolerance probably involves a system other than HSP70. Factors that may be essential for embryonic induction of thermotolerance may include antioxidants, growth factors and amino acids.

In Chapter VI, an experiment was described in response of cooling heat-stressed cows from 2 to 3 d before breeding until 5 to 6 d after breeding on pregnancy rates. This short-term cooling scheme was successful in improving pregnancy rates during summer months in Florida. However, the regimen was not able to restore pregnancy rates to that observed during winter months. Previous studies in which cows were cooled for the first 4 to 10 d after breeding reported a great (Gauthier, 1983), slight (Stott and Wiersma, 1976) or no beneficial effect (Her *et al.,* 1988) on pregnancy rates during summer months. It was anticipated that the cooling system used in Chapter VI would be more successful than previous experiments because cooling was initiated before estrus, when final follicular

development and oocyte maturation occurs. Failure to achieve optimal pregnancy rates implies that heat stress before or after the cooling period had detrimental effects on embryonic development. Follicular development is altered during heat stress in cattle (Badinga *et al.,* 1993), suggesting that this detrimental effect may have contributed to some of the pregnancy losses. Future experiments are required to develop a short term cooling system which will be beneficial for commercial use to prevent heat stress effects on pregnancy rates.

The antioxidants GSH and taurine were examined for their ability to protect embryos from deleterious effects of heat shock in Chapter VII. At the morula stage, GSH and taurine were capable of partially protecting bovine embryos from heat shock effects. In contrast, GSH, monoethyl GSH ester, and taurine were not successful in protecting 2-cell bovine embryos from adverse effects of heat shock. Therefore, it appears that GSH and taurine are not capable of protecting embryos during periods of development when embryos are highly sensitive to effects of elevated temperatures. Antioxidants may not be effective at the 2-cell stage since they have not developed mechanisms which are necessary to aid in eliciting thermoprotective effects of antioxidants. It is also possible that different dosage or timing of administration relative to heat shock is required at earlier stages.

In conclusion, these studies demonstrate that murine and bovine embryos gain resistance to elevated temperature as development progresses. The increased resistance appears to be caused by increased resistance of embryos to direct effects of elevated temperature. Provision of intensive cooling during final oocyte

maturation and early embryonic development improved pregnancy rates of cows during summer months in Florida, but effects were not extreme. This suggests that additional periods of oocyte and embryonic development are also highly susceptible to heat stress effects. Thermoprotection of cultured embryos can be achieved by administration of GSH and taurine but such antioxidant therapy was not beneficial at the 2-cell stage.

Findings presented in this dissertation conclusively demonstrate that embryos gain resistance to heat as development progresses. Future work is required to identify intracellular mechanisms which confer thermal resistance for embryos. With this knowledge, additional regimens can be developed for reducing heat stress effects on pregnancy rate in dairy cows. The use of short-term cooling has been shown to be a promising scheme for reducing heat stress effects on pregnancy rate. This system may be more effective if cooling is provided for an extended period preceding breeding (7 to 10 before breeding), when growth and dominance of the follicle destined to ovulate occurs. Additional research is required to determine the efficacy of antioxidant therapy for protecting embryos from heat. Future studies should concentrate on identifying molecules which are thermoprotective at early stages of development.

APPENDIX A
BOVINE *IN VITRO* MATURATION/FERTILIZATION/CULTURE TECHNIQUES

Procedures

These procedures are modifications of existing procedures of Parrish *et al.* (1988), Xu *et al.* (1992) and Hernandez-Ledezma *et al.* (1993).

Warming Culture Room

1. On the morning of use, warm the inner culture room to at least 30 C.

2. Seal the air-conditioning vent with card-board, turn on the laminar flow hood and turn on the space heater. After 3 to 4 h the room will be warm enough to work.

3. The air-conditioning vent can be sealed for extended periods of time if IVF is conducted routinely.

BRLC Culture

1. Store Buffalo Rat Liver Cells (BRLC; American Type Culture Collection; BRL-3A; TCC CRL 1422) in the liquid nitrogen tank until use.

2. To begin BRLC culture, remove 1 vial of cells from liquid nitrogen and place in warm water (37 to 40 C) for 40 to 60 seconds. Dip vial into 70% ETOH and place contents in a T25 flask (Corning, #25106). Add 10 ml of Hams-F12 medium (containing 5% BSS and 1% Pen/Strep), lay flask on its side, loosen lid and incubate for 24 h at 37 C.

3. After 24 h, examine cells under the reverse phase microscope; cells should have attached to the bottom of the flask and be 30 to 70% confluent. Remove medium and replace with 10 ml Hams-F12 containing additives. Medium must then be changed every 48 to 72 h.

Note: Percent confluency is a subjective scoring system to determine whether a monolayer of cells have formed. A complete monolayer, or 100% confluency, is defined as a single layer of cells covering the entire bottom of the flask.

4. Once BRLC have become 100% confluent (2 to 6 d), sub-culture by removing medium and adding 1 to 2 ml trypsin (Stock 23). Incubate cells at 37 C for 5 min for trypsin to detach cells from the flask.

5. Following incubation, cells still remaining attached to the flask must be removed by manually scraping off cells using a cell scraper (Fisher #08-773-2) or plastic pipette. Place cells (still in trypsin) from each flask into 3 to 4 new T25 flasks, add 10 ml of Hams-F12 plus additives to each flask and incubate at 37 C as described previously (as if freshly thawed cells).

6. Cells should not be discarded unless they become contaminated or ample quantities of BRLC exist (in culture or frozen). Instead, continuously grow and sub-culture cells for use in IVF procedures or freeze to replenish BRLC stocks.

7. If cells are discarded, remove medium and treat flasks with bleach for 5 minutes (to kill cells).

8. Freeze BRLC by detaching cells with trypsin, centrifuging at 1500 rpm for 15 min and adding 1 ml Hams F12 (plus 5% BSS and pen/strep) containing 5% DMSO for each flask of cells to be frozen. Aliquot cell suspension in 1 ml volumes to cryo-store tubes (Dynatech, #006-010-0101) and store at -70 C overnight. Place cells into a styrofoam container to reduce the quickness of freezing. The following morning, place frozen cells into liquid nitrogen.

Conditioning Media

1. BRLC-conditioned medium is preferred over conditioning with primary cells because of decreased incidence of contamination. Condition medium with BRLC when confluency is between 50% to 100%. However, the greater the confluency, the greater the level of medium conditioning.

2. To condition medium (CZB medium or LEC medium); remove Hams-F12 medium from one flask containing BRLC, wash with 10 ml of the desired medium, then place 10 ml of fresh medium into flask and incubate for 24 to 48 h.

3. Following conditioning, remove medium from flask and sterile filter. Medium can be used immediately or stored at 4 C for up to 7 d before use.

4. Following BRLC-conditioning, cells can be re-used either to condition additional medium or for sub-culture.

Oviduct Cell Co-Culture

1. Collect 1 to 2 oviducts from peri-estrous tracts still attached to ovaries and transport with ovaries to the laboratory.

2. Upon return to the lab, dissect off all tissues from oviducts except the tip of the uterine horn. Cut off the fimbrial portion of oviducts, swab with 70% ETOH and place into a 60 x 15 mm culture dish containing oviduct washing medium in the laminar flow hood.

3. Squeeze oviducts from the isthmus end (uterotubular junction) with small forceps into a second 60 x 15 mm culture dish containing oviduct washing medium (<15 ml).

4. Aspirate oviduct cells once using a 1 ml syringe and 25 ga. needle. Transfer medium containing cells to a 15 ml conical tube and place upright at 39 C for 3 to 5 min.

5. Discard supernatant and wash cells by adding 5 to 10 ml oviduct washing medium, titrating cells and allowing cells to settle at 39 C for 3 to 5 min. Repeat wash steps 2 to 3 times. Smaller debris should be discarded using this washing process.

6. After washes, distribute cells into 6-well plates so that oviduct cells encompass approximately 30% of each well's surface. Add 4 to 5 ml of oviduct culture medium and incubate for up to 7 d at 39 C.

7. Oviduct cells can be used to either condition medium or as a source of cells for co-culture.

8. To condition medium, place 5 ml of medium to be conditioned with oviduct cells for 24 to 48 h. Remove medium and sterile filter. Medium can be used immediately or stored at 4 C for up to 7 d.

9. For co-culture, remove oviduct cells from 1 well, place in a 15 ml conical tube and allow cells to settle for 5 to 10 min at 39 C. Wash once with medium to be used (as described earlier). Add medium to preparation to yield 10 to 15 clumps of oviducts per 50 μl drop of medium. Incubate at 39 C for at least 18 h before adding oocytes or embryos.

Collection of Ovaries and Retrieval of Oocytes

1. Before leaving for the slaughterhouse, place all media to be used for oocyte collection at 39 C (except for saline; store at room temperature). Place 7 ml of oocyte maturation medium in 60 x 15 mm culture dishes and incubate at 39 C.

2. Place 4 L saline and 50 ml ABAM (Stock 18) into each thermos. Take thermos, knives, hair-net, hard-hat, gloves, boots and lab-coat to slaughterhouse.

3. Remove ovaries from reproductive tract of cows immediately after internal organs are extracted from the carcass and place into one thermos. Collect only ovaries with substantial follicular development.

4. After ovary collection, transfer ovaries from the first thermos into the second and transport to the lab, immediately.

5. Upon return to the lab, wash ovaries 3 to 4 times with fresh transport saline. Following washes, place ovaries in beakers containing saline and store at room temperature until time of oocyte collection.

6. Add 100 ml oocyte collecting medium into 250 ml beakers.

7. Attach a hemostat to the base of the ovary such that the ovary is firmly held in place. Discard fluid of large follicles (>10 mm) by rupturing and discarding fluid before processing ovaries (follicular fluid promotes clotting of medium). Hold ovary above beaker and make checkerboard incisions (for 2 to 3 mm squares) across entire ovary. To prevent contamination of medium with blood (which promotes formation of clots), do not make checkerboard incisions across corpora lutea.

8. Submerge ovary into oocyte collecting medium and swirl vigorously. Repeat process until the group of 8 to 15 ovaries has been processed.

9. Once completed, place beaker at room temperature (at least 30 C) for 5 to 10 minutes to allow oocytes and debris to settle. Using a 25 ml pasteur pipette, remove all but the bottom 25 to 50 ml of medium. Add 100 ml of fresh oocyte collecting medium and repeat process until medium is clear. Remove all but the bottom 25 to 50 ml of medium, then transfer into a gridded culture dish (100 x 15 mm; Falcon 1012) and place on plate warmer.

10. Collect oocyte cumulus complexes (OCC) as fast as possible to prevent adverse effects of cold shock. Only OCC which have at least one layer of compact cumulus cells and an evenly granulated cytoplasm with no dark spots or clear

spaces should be used for subsequent steps. Place OCC retrieved into one 60 x 15 mm culture dish containing HEPES-TALP on the plate warmer.

11. After oocyte retrieval is completed for each dish, transfer oocytes to a dish containing oocyte maturation medium. It is essential that oocytes be collected, washed and incubated in oocyte maturation medium as quickly as possible.

12. Place no more than 250 OCCs in each dish (60 x 15 mm) containing 7 to 10 ml oocyte maturation medium and incubate for 24 to 28 h at 39 C.

In Vitro Fertilization

1. Prepare several 4-well plates (Nunc, #176740) with 600 μl IVF-TALP and incubate (39 C) until use.

2. Place HEPES-TALP into several culture dishes (60 x 15 mm) and place on plate warmer until use.

3. Remove one to two dishes containing matured oocytes, retrieve OCC and place oocytes in HEPES-TALP. Wash once in HEPES-TALP.

4. Following washes, place 20 to 40 oocytes per well in IVF-TALP. Repeat process for additional dishes until all oocytes are processed.

5. After all oocytes are in IVF-TALP, add 25 μl sperm suspension and 25 μl PHE mix (Stock 20). One well should be prepared without sperm, but with PHE, to determine the incidence of parthenogenesis. Incubate for 14 to 18 h at 39 C.

6. Use the DAPI staining procedure to determine rate of oocyte maturation and/or fertilization.

Sperm Preparation

Note: It is critical that spermatocytes not be exposed to cold shock during the following steps; make sure that all media used during these steps are warmed to 39 C before use.

Percoll gradient:

This method for isolating viable sperm is much more efficient than sperm swim-up.

1. Place 6 ml of 90% Percoll in two 15 ml conical tubes.

2. Slowly layer 6 ml of 45% Percoll on top of the 90% for both tubes; incubate gradient at 39 C for at least 1 h.

3. Thaw 4 to 6 straws of semen from several bulls in the cyto-thaw for 60 seconds.

4. Slowly add 1/2 of the thawed semen to the top of each of the Percoll gradients. Centrifuge at 2500 rpm for 35 min using the IEC centrifuge.

5. After centrifugation, collect sperm pellet at the bottom of the Percoll gradient. Place in a 15 ml conical tube containing 10 ml SP-TALP. Centrifuge at 1000 rpm for 5 min.

6. Discard all but the bottom 200 to 500 μl of supernate.

7. Add 10 μl sperm suspension to 90 μl ddH_2O (kills sperm). Load 10 μl of sample onto a hemocytometer. Count the number of sperm in 5 squares. Multiply sperm number by 500,000 to determine concentration per ml. Adjust final concentration to approximately 25 X 10^6 sperm/ml with IVF-TALP.

8. Add 25 μl sperm preparation (for ~1 x 10^6 sperm/ml) and 25 μl PHE mix (Stock 20) to each droplet (except parthenogenesis drops) and incubate at 39 C for 14 to 18 h.

Swim-up:

1. Thaw 6 to 8 straws of frozen semen in the cyto-thaw for 60 seconds. If possible use semen from different bulls.

2. Combine contents of straws in 5 ml Sp-TALP. Place sample into the incubator (39 C) for 5 minutes.

3. Centrifuge semen (1000 rpm; 5 min) and discard all but the bottom 1 ml of supernatant.

4. Prepare 4 to 5 test tubes containing 1 ml Sp-TALP. Add approximately 250 μl of sperm suspension very slowly to the bottom of each tube using a 20 ga. needle and 1 ml syringe. Place tubes in incubator (39 C) for 1 h.

5. At the end of sperm swim-up, aspirate the top 800 μl from each tube and combine samples and centrifuge (1000 rpm) for 5 minutes. Discard all but the bottom 500 μl of supernate.

6. Add 10 μl sperm suspension to 90 μl ddH_2O (kills sperm). Load 10 μl of sample onto a hemocytometer. Count the number of sperm in 5 squares (see

laboratory materials and methods book). Multiply sperm number by 500,000 to determine concentration per ml. Adjust final concentration to approximately 25 X 10^6 sperm/ml with IVF-TALP.

7. Add 25 μl sperm preparation (for ~1 x 10^6 sperm/ml) and 25 μl PHE mix (Stock 20) to each droplet (except parthenogenesis drops) and incubate at 39 C for 14 to 18 h.

Post-IVF: Early Embryonic Development

1. On d 1 post-IVF, prepare several 4-well plates containing 600 μl BRLC-conditioned CZB medium and incubate (39 C) until use.

2. Transfer oocytes/embryos from fertilization medium of one plate into a 60 x 15 mm dish containing HEPES-TALP (to wash oocytes/embryos).

3. Collect OCC from HEPES-TALP, place in a 15 ml conical tube containing 1 to 2 ml medium and vortex for 1 min (to remove cumulus cells and attached sperm). Wash oocytes twice in HEPES-TALP.

4. Place 50 to 60 oocytes/embryos into conditioned CZB medium and incubate at 39 C for 48 h. Repeat process until all oocytes/embryos have been processed.

> *Note: It is essential that oocytes/embryos be transferred as quickly as possible.*

Late Embryonic Development

1. On d 3 post-IVF, transfer 4-cell, 8-cell and 16-cell embryos into BRLC-conditioned LEC medium. Again, it is essential that embryos be transferred as quickly as possible.

Alternative Embryo Culture Procedure

1. On d 1 post-IVF, prepare 50 μl drops of BRLC-conditioned LEC medium (may also use oviduct cell-conditioned or oviduct cell co-culture methods) in 60 x 15 mm culture dishes, cover with paraffin oil and incubate at 39 C for at least 1 h.

2. Transfer 20 vortexed oocytes/embryos into drops and incubate at 39 C.

3. On d 3 post-IVF, add 50 μl BRLC-conditioned LEC medium to each drop and incubate at 39 C to the desired stage of development.

Preparation of Media

General Notes

1. When making a solution, please place two dates on the bottle if appropriate: the date made and the expiration date. Additionally, label all stocks "FOR IVF USE".

2. Store all media at designated areas in the walk-in cooler (4 C) or the laboratory freezer (-20 C).

3. Unless otherwise noted, use Sigma culture grade water.

4. In general, place medium in oven 1 to 2 h before use. However, if medium is placed in culture dishes, place medium in incubator (to maintain pH).

5. For stocks, label every tube.

6. Don't sterile filter solutions if you can autoclave. When autoclaving solutions, mark the volume of each container before autoclaving. After autoclaving, bring the volume of solutions back to their original volume with autoclaved water.

7. During filtration, use the most economical filters for the volume being sterilized. If filtering less than 20 ml, use syringe tip filters. For larger volumes, screw-on bottle filters are the most economical. Generally, filters can be used to sterilize several types of media.

8. Soak all glassware in either Sigma-Clean or PCC-54 baths before use. It is essential that all glassware be cleaned in this way for optimal results.

Stock Solutions

Stock 1: NaCl. Dissolve 13.33 g in 100 ml water. Sterile filter and store at 4 C. Label as "Stock 1: NaCl; date made".

Stock 2: KCl. Dissolve 0.588 g in 50 ml water. Sterile filter and store at 4 C. Label as "Stock 2: KCl; date made".

Stock 3: Bicarb. Dissolve 1.052 g $NaHCO_3$ in 50 ml water. Sterile filter and store at 4 C for one wk only. Label as "Stock 3: Bicarb; date made; expiration date".

Stock 4: PO_4. Dissolve 0.235 g $NaH_2PO_4+H_2O$ in 50 ml water. Sterile filter and store at 4 C. Label as "Stock 4: PO_4; date made".

Stock 5: Na lactate. Purchase as a 60% syrup. Store indefinitely at 4 C. Label as "Stock 5: Na Lactate; date purchased".

Stock 6: 1 M HEPES. Add 119 g of HEPES to 400 ml water. Adjust pH to 7.0 and bring volume up to 500 ml. Autoclave and store at 4 C indefinitely. Label as "Stock 6: 1 M HEPES; date made".

Stock 7: $CaCl_2$. Dissolve 1.470 g $CaCl_2+2H_2O$ in 50 ml water. Sterile filter and store at 4 C. Label as "Stock 7: $CaCl_2$; date made".

Stock 8: $MgCl_2$. Dissolve 1.017 g of $MgCl_2+6H_2O$ in 50 ml water. Sterile filter and store at 4 C. Label as "Stock 8: $MgCl_2$; date made".

Stock 9: Pyruvate. Dissolve 0.220 g sodium pyruvate in 100 ml water. Sterile filter into an aluminum-foil wrapped 125 ml bottle and store at 4 C for 2 mo. Label as "Stock 9: Pyruvate; date made; expiration date".

Stock 10: Bovine Steer Serum (BSS). Prepare 10 ml aliquots of BSS (Pel-Freeze) and store at -20 C indefinitely. Label as "Stock 10: BSS; date made".

Stock 11: BSS/Hep. Add 1000 USP units of sterile sodium heparin (dissolved in small volume of water) into 500 ml BSS. Store in 8 ml aliquots indefinitely at -20 C. Label as "Stock 11: BSS/Hep; date made".

Stock 12: Estradiol. Dissolve 1 to 3 mg estradiol in ethanol for a final concentration of 1 mg/ml. Store in a glass tube at -20 C for 2 mo. Label as "stock 12: Estradiol; date made; expiration date".

Stock 13: FSH-P. Reconstitute FSH-P with saline for a final concentration of 5 mg/ml (follow directions on bottle). Place 500 μl aliquots into sterile 1.5 ml microfuge tubes and store indefinitely at -20 C. Label as "stock 13: FSH-P; date made".

Stock 14: Heparin. Dissolve 20 mg sodium heparin in 10 ml water. Pipette into 250 μl aliquots and store at -20 C in bullet tubes indefinitely. Label as "Stock 14: Hep; date made".

Stock 15: Gentamicin. (Sigma, G-1397; 50 mg/ml). Dilute to 5 mg/ml with water and sterile filter. Pipette 1 ml aliquots into sterile 12 x 75 tubes and store at -20 C indefinitely. Label as "Stock 15: Gent; date made".

Stock 16: ABAM (1 ml). (Sigma, A-9909). Aliquot 1 ml into sterile 12 x 75 tubes and store indefinitely at -20 C. Label as "Stock 16: ABAM (1 ml); date made".

Stock 17: ABAM (4 ml). Aliquot 4 ml into sterile 12 x 75 tubes and store at -20 C indefinitely. Label as "Stock 17: ABAM (4 ml); date made".

Stock 18: ABAM (for transport saline). Combine 0.555 g Amphotericin B (Solubilized; Sigma, A-9528; 25 μg/ml), 100 million IU Penicillin G (Sigma, P-3032; 10,000 IU/ml), and 100 g Streptomycin Sulfate (Sigma, S-9137; 10 mg/ml) with 10 L saline and allow for complete solubilization by prolonged stirring. Sterile filter or autoclave and store in 50 ml conical tubes in walk in freezer. Label as "Stock 18: Home-made ABAM (100x)".

Stock 19: Hyaluronidase. Prepare stock solution of type IV hyaluronidase at 10 mg/ml saline (approx. 8000 U/ml) and store 1.2 ml aliquots at -20 C indefinitely. Label as "Stock 19: Hyal.; date made".

Stock 20: PHE Mix. Prepare primary stocks of 1 mM hypotaurine (Sigma H-1348; 1.09 mg/10 ml saline), 2 mM penicillamine (Sigma P-5000; 3 mg/10 ml saline) and 250 μM epinephrine (Sigma E-4250; 1.83 mg/ 40 ml of the following solution (165 mg 60% Na lactate syrup, 50 mg Na metabisulfate and 50 ml H_2O)). Epinephrine is easily oxidized by direct light so take precautions to avoid this problem. Combine 5 ml of 1 mM hypotaurine, 5 ml of 2 mM penicillamine, 2 ml of 250 μM epinephrine and 8 ml of saline and sterile filter. Aliquot 400 μl of PHE Mix into sterile microfuge tubes and store in a light resistant container at -20 C indefinitely. Upon retrieval of PHE Mix for use, wrap tube in aluminum foil. Label as "Stock 20: PHE mix; date made".

Stock 21: Glutamine. Prepare stock solution of 1.5 g glutamine/ 100 ml water, sterile filter and store 1 ml aliquots at -20 C indefinitely. Label as "Stock 21: Glutamine; date made".

Stock 22: $MgCl_2$ for Percoll. Prepare 0.1 M stock by adding 0.0203 g $MgCl_2$ to 10 ml water. Sterile filter and store at 4 C indefinitely. Label as "Stock 22: $MgCl_2$ for Percoll; date made".

Stock 23: $CaCl_2$ for Percoll. Prepare 1 M stock by adding 0.735 g $CaCl_2+2H_2O$ to 5 ml water. Sterile filter and store at 4 C indefinitely. Label as "Stock 23: $CaCl_2$ for Percoll; date made".

Stock 24: Trypsin. Prepare 5 ml aliquots of 1x trypsin (Sigma, #T-5650) and store at -20 C indefinitely. Label as "Stock 24: Trypsin; date made".

TCM-199 stock medium

Water	800 ml
TCM-199 (Earle's salts; powder) (Sigma, M-5017)	for 1 l
$NaHCO_3$	2.2 g

1. Adjust pH to 7.2 and bring volume to 1 l. Sterile-filter 90 ml aliquots into opaque or aluminum-foil wrapped 100 ml bottles and store at 4 C for 2 mo. Label as "TCM-199 Stock Medium; date made".

2. Beginning 2 wk after TCM-199 stock medium is made, 1 aliquot of stock 21 (glutamine) is need to replenish glutamine that has degraded.

3. Alternatively, liquid medium can be purchased from Sigma (M-4530) or Fisher (MT-10-060-LV).

Oocyte Collecting Medium

Water (use ddH_2O)	9 L
TCM-199 (-Phenol red, -Glut.) (Sigma, M-3274)	For 10 L
$NaHCO_3$	3.50 g
Glutamine	1.34 g
Stock 6: 1 M HEPES	100 ml

1. Adjust pH to 7.2 and bring volume to 10 L. Sterile-filter 400 ml medium into 500 ml bottles and keep indefinitely at 4 C. Labels should read "Oocyte Collecting Medium, -BSS -ABAM - Hep; date made".

2. On night before use, add 1 aliquot of stock 11: BSS + Hep and 1 aliquot of stock 17: ABAM (4 ml) to one bottle. Change label to + BSS + Heparin + ABAM and use within 2 wk. Write expiration date on label. Generally, 3 to 5 bottles are needed on d of oocyte collection.

3. If collecting medium is more than 2 wk old, add 4 ml stock 21: glutamine per bottle.

Oocyte Maturation Medium

1. On night before use, add 1 aliquot stock 10: BSS, 1 aliquot stock 15: gent., 400 μl stock 13: FSH-P, 200 μl stock 12: estradiol, 1 ml stock 9: pyruvate and 1 ml stock 21: glutamine if needed to one bottle of TCM-199 (90 ml). Store at 4 C

for up to 7 d. Change label to read "Oocyte maturation medium; expiration date".

CZB medium: Early Embryo Culture Medium

1. Prepare a stock containing the following:

Ingredient	g/0.5 liter	g/liter
NaCl	2.385	4.770
KCl	0.180	0.360
KH_2PO_4	0.080	0.160
$NaHCO_3$	1.055	2.110
EDTA	0.016	0.032
$MgSO_4+7H_2O$	0.145	0.290
$CaCl_2+H_2O$	0.125	0.250
Phenol Red	0.005	0.010

2. Adjust pH for 7.3 and sterile filter 88 ml of CZB medium into 100 ml bottles and store at 4 C indefinitely. Label as "CZB medium; -Suppl; date made".

3. On d before use add 0.45 ml stock 5: Na lactate, 1 ml stock 9: pyruvate, 1 ml stock 21: Glutamine, 1 aliquot stock 15: Gent and 1 aliquot stock 10: BSS. Store at 4 C for up to 14 d. Change label to read "CZB medium; + Suppl.; expiration date".

Late Embryo Culture (LEC) Medium

1. Add 1 aliquot stock 10: BSS, 1 aliquot stock 15: gent. and 1 ml stock 9: pyruvate to 90 ml TCM-199. Store at 4 C for up to 14 d. Change label to read "LEC Medium; expiration date".

3. If TCM-199 stock is more than 2 wk old, add 1 ml stock 21: glutamine to bottle.

TL Media

Ingredient (ml)	Sp-TL	HEPES-TL	IVF-TL
Water	79.232	176.0	40.157
Stock 1: NaCl	4.34	11.0	2.5
Stock 2: KCl	1.96	4.0	1.0
Stock 3: Bicarb	10.0	1.6	5.0
Stock 4: PO_4	1.0	2.0	0.5
Stock 5: Na lactate	0.368	0.372	0.093
Stock 6: HEPES	1.0	2.0	0
Stock 7: $CaCl_2$	1.0	2.0	0.5
Stock 8: $MgCl_2$	1.1	1.0	0.25
pH	7.4	7.3	7.4
Osmolarity (mOsm)	295-305	275-285	290-300

1. Make up every wk. Adjust pH on solutions and check osmolarity. If osmolarity is not correct, discard solutions and start over. Use TL stocks to prepare TALP solutions. Sterile filter remaining SP-TL and store at 4 C for up to 1 wk. Write expiration date of label.

TALP Media

Ingredient	Sp-TALP	HEPES-TALP*	IVF-TALP*
TL (ml)	38	200	50
BSA, Fraction V (mg)	240	600	0
BSA, EFAF (mg)	0	0	300
Stock 9: Pyruvate (ml)	2	2	0.5
Stock 15: Gent. (μl)	80	300	50
Stock 14: Heparin (μl)	0	0	250

* Use entire volume of TL stocks to prepare TALP solutions

1. Sterile filter and store at 4 C until use. Discard after 1 wk.

90% Percoll

1. Purchase Percoll from Sigma (#P-1644) and store at 4 C until use.

2. Prepare 10x SP-TL stock solution:

Chemical	g/100 ml H2O
NaCl	4.675
KCl	0.23
$NaH_2PO_4+H_2O$	0.40
HEPES	2.38

3. Adjust pH to 7.3, sterile filter and store indefinitely at 4 C. Label as "10x SP-TL stock; for Percoll; date made".

4. To prepare 90% Percoll solution (on d of use), place 2 ml of 10x SP-TL to a small beaker and add 0.042 g bicarbonate and 74 μl Na lactate (Stock 5). After bicarbonate and lactate dissolve, add 18 ml Percoll and stir.

5. While stirring, add 79 μl $MgCl_2$ (stock 22) and 39 μl $CaCl_2$ (stock 23).

6. Adjust pH to 7.3-7.45 and filter with a .45 μm syringe filter. If compounds precipitate out of the percoll solution, continue to stir. If compound do not re-dissolve, then start over.

7. Prepare the 45% percoll solution by adding equal volumes of 90% percoll solution and SP-TL (1x). Mix thoroughly.

Hams-F12 Medium

1. Prepare Hams-F12 medium (Sigma, #N-6760) by adding powder to ddH_2O (Simmen's Lab). Either 1 or 5 L of can be prepared, depending on rate of use. Add 1.176 g/l bicarbonate to solution, pH to 7.2 - 7.3 add bring up to the desired volume. Sterile filter into 100 ml or 500 ml bottles (again, depending on rate of use) and store at 4 C for up to 2 mo.

2. On d of use, add stock 10: BSS for a final concentration of 5% and 1% pen/strep. Use within 2 wk.

3. If Hams-F12 stock is greater than 2 wk old, add 1 ml stock 21: glutamine/ 100 ml medium.

Transport Saline

1. Add 90 g NaCl to 10 l distilled water and autoclave. Label as "Sterile 0.9% saline; date made" and store indefinitely at room temperature.

2. Place 4 l saline and 50 ml stock 18: ABAM into each thermos on morning of use. Save remaining saline for washing ovaries following their transport to the laboratory.

Paraffin Oil

1. Purchase embryo tested mineral oil from Sigma (M-8410) and store at room temperature indefinitely.

2. Alternatively, paraffin (VWR, #S894-07) or silicon (Aldrich, #14,615-3) oil that has not been embryo tested can be used if first extracted with water. Combine equal volumes of oil and water and incubate at 37 to 40 C overnight. Repeat process two to three times and store oil at room temperature. Since toxic effects of these oils may still persist following extraction, use of embryo tested mineral oil from Sigma is preferred.

Additional Media for Oviduct culture

Note: These media do not need to be prepared unless oviduct cells are being used.

1. Oviduct Washing Medium: Combine 1 aliquot stock 10: BSS, 1 aliquot stock 16: ABAM (1 ml), 220 μl Na lactate (stock 5) and 1 ml pyruvate (Stock 9) to 90 ml TCM-199 on night before use. Store at 4 C and use within 2 wk. If TCM-199 is more than 2 wk old, add 1 ml glutamine (Stock 21) to bottle.

2. Oviduct Culture Medium: Combine 1 aliquot stock 10: BSS, 1 aliquot stock 16: ABAM and 1 ml stock 9: pyruvate. Store at 4 C and use within 1 wk. If TCM-199 is more than 2 wk old, add 1 ml glutamine (Stock 21) to bottle.

DAPI stain

1. Prepare PBS stock containing 3% glutaraldehyde (v/v; 3 ml glut./ 100 ml PBS). Store indefinitely at room temperature in opaque or foil wrapped bottle.

2. Prepare DAPI stock by adding 0.1 mg DAPI (Sigma, #D-9542) to 10 ml sterile saline (0.001% DAPI). Store at 4 C in foil wrapped culture tube for up to 4 mo.

3. Add 200 μl stock 19: hyal. to 800 μl maturation medium in microfuge tubes and place at room temperature.

4. Remove 10 to 20 oocytes from washing steps after maturation or fertilization and place in hyaluronidase solution for 2 to 3 min and centrifuge (via microfuge) for 1 min.

5. Fix oocytes with 3% glutaraldehyde in a 35 x 10 mm culture dish at room temperature for 15 minutes.

6. Wash oocytes in PBS and transfer to 0.001% DAPI solution (in smallest volume possible; 500 μl). Incubate at 37 C for 10 minutes.

7. After DAPI staining, place oocytes on slides with a small volume of PBS (5 to 10 μl). DAPI will bind DNA and emit fluorescence with a 490 nm emission filter.

8. Oocytes have matured through meiosis (as desired) if staining is seen in a small area within the oocyte and within 1 polar body.

9. Oocytes have been fertilized when 2 pronuclei are evident.

"Check-list"

----------Day -2 to -3----------

1. Prepare necessary stock solutions and media.

2. Begin BRLC culture.

----------Day-1----------

1. Contact slaughterhouse to ensure that cows will be slaughtered.

2. Prepare transportation to slaughterhouse.

3. Prepare transport saline.

4. Prepare following solutions if needed:
   -oocyte maturation medium (1 bottle)
   -oocyte collection medium (6 to 7 bottles)
   -TALP solutions (1 bottle of each)

5. Gather all materials needed for the trip to the slaughter house:

thermos	knives	scissors
hair-net	hard hat	gloves
boots	lab coat	

----------Day 1----------

Before departure:

1. Place saline and ABAM into two thermos.

2. Place 7 ml Oocyte Maturation Medium in 60 x 15 mm dishes.

3. Warm the following media in oven (39 C):
   -Oocyte collection medium (3 to 5 bottles)
   -HEPES-TALP

4. Warm culture room.

Upon return:

1. Rinse ovaries with remaining saline.

2. Gather materials for oocyte collection.

    hemostats
    scalpel blades and handles
    scissors
    container (1 l)
    250 ml Beakers

3. Collect and search for oocytes:
    -Wash oocytes with HEPES-TALP
    -Transfer to oocyte maturation medium (incubate for 24 h)

4. Begin BRLC-conditioning of CZB or LEC medium.

----------Day 2----------

1. Warm culture room.

2. Prepare Percoll.

3. Warm water in Cyto-thaw.

4. Warm HEPES-TALP and IVF-TALP.

5. Begin to isolate viable sperm with percoll gradient.

6. Wash and transfer oocytes to IVF-TALP.

7. Isolate and add sperm (and PHE mix) to oocytes (incubate for 14 to 18 h).

----------Day 3----------

1. Warm culture room.

2. Warm HEPES-TALP and incubate BRLC-conditioned CZB medium or BRLC-conditioned LEC medium at 39 C.

3. Wash and transfer oocytes/embryos to desired medium (incubate for 48 h).

4. Begin BRLC-conditioning of LEC medium.

----------Day 5----------

1. Warm culture room.

2. Incubate BRLC-conditioned LEC medium at 39 C.

3. Transfer ≥4-cell embryos to BRLC-conditioned late embryo culture medium or add BRLC-conditioned LEC medium to drops, depending on culture method.

APPENDIX B
MOUSE SUPEROVULATION, EMBRYO COLLECTION AND CULTURE

Purchasing and Housing Mice

1. Purchase C57Bl/6J, CBA/J, or B6CBAF$_1$/J (C57 x CBA) mice from Jackson Laboratories or ICR random bred mice from Charles Rivers. House mice at the Animal Science Nutrition Lab at a photoperiod schedule of 14 h light: 10 h dark. Set the light timer for lights to go off at 1900 h and on at 0500 h (midpoint of the dark cycle is midnight).

2. For best response to superovulation, females should be between 21 to 30 d of age. For mice greater than 35 d of age, breed naturally, they will not respond well to superovulation.

3. Males should be post-pubertal (40 to 50 d old). ICR males are the best males to use. Place males in individual cages for 1 wk before use. Only use males twice a wk for breeding (on non-consecutive d). Males should perform well for up to one year, however, replacing males every 9 mo is advised.

4. Do not allow mice to accumulate in the room, dispose of mice not to be used for experiments by placing them in the Veterinary School Pathology cold room with an incineration tag.

Superovulation

Hormone Preparation

1. Hormones to be used for superovulation can be purchased from several companies. Sigma products have been the most successful for this lab.

2. Prepare PMSG stock by solubilizing 100 IU PMSG (Sigma, G-8777)/ 10 μl sterile saline. Place 10 μl aliquots of stock to 1.5 ml sterile microfuge tubes and store at -20 C for up to 2 mo. On d of use add 990 μl sterile saline (for a concentration of 10 IU/ 100 μl).

3. Prepare hCG stock by solubilizing 75 IU hCG (Sigma, CG-5)/ 10 μl sterile saline. Place 10 μl aliquots of stock in 1.5 ml sterile microfuge tubes and store at -20 C indefinitely. On d of use add 990 μl sterile saline (for concentration of 7.5 IU hCG/ 100 μl saline).

Injection and Breeding Schedule

1. Inject 10 IU PMSG (100 μl) intraperitoneally with a 30 ga x 1/2" needle and 1 ml syringe into female mice between 1500 and 1700 h.

2. After 43 to 47 h (1200 to 1400 h), inject mice with 7.5 IU hCG (100 μl) intraperitoneally. Timing of hCG injection is crucial for maximal superovulatory response, it must occur before 1400 h.

3. Following hCG administration place females with males overnight. Housing 1 female per male gives optimal responses but 2 to 3 females can be placed with a male if necessary.

4. Check females for the presence of a vaginal plug between 0600 and 1200 h (ideally 0800 h) on the morning after hCG treatment. Vaginal plugs can be observed as a clear to cream colored solid mass located in the vulva/vagina. Presence of the plug indicates that copulation has occurred over the preceding night (approximately at the midpoint of the dark cycle).

5. Only use females with vaginal plugs for embryo collection and culture procedures.

Embryo Collection

1. Embryos can be collected from mouse oviducts up to 3 d after hCG and from mouse uterine horns 4 d after hCG. Unless one cell embryos are desired, the ideal time to collect embryos is 2 d after hCG (2-to 4-cell stages). This is because oviducts are easier to flush, embryo yield is greatest and embryo searching is easier compared with other times.

2. Prepare all materials for flushing and embryo collection. Organize the dissecting microscope, M2 medium containing 0.4% BSA (two 35 x 10 mm culture dishes; 1 dish containing grids, 1 dish without grids), one paper towel, one 30 ga x 1/2" needle and one 1 ml syringe in an area that can be easily cleaned. Also, place 2 small forceps and 1 pair of small scissors in a beaker containing 70% ETOH.

3. Kill mice by cervical dislocation; place a pencil behind the head and pull the body away from the head by pulling on the tail.

4. Rinse the genitals and abdomen with 70% ETOH and place mice on paper towel.

5. Expose the reproductive tract by cutting the lower abdominal skin and peritoneum. Move the intestines out of the lower abdominal region to find the tract.

6. Grab and retract one of the uterine horns and grossly dissect away connective tissue. Cut tissue anterior to the ovary and in the middle of the uterine horn. Place tissue (ovary, oviduct and part of the horn) in culture dish without grids containing M2 medium. Repeat process for other horn.

7. After oviducts have been collected from all mice, transfer tissues into culture dish with grids containing M2 medium.

8. Dissect out oviducts from ovaries and horns. Remove ovaries and horns from culture dish.

9. Grossly dissect the ampulla end of oviducts (proximal to the uterine horn) from the rest of the oviduct using two pairs of forceps. The ampulla end can be identified by the presence of blood vessels. This end of the oviduct is generally much easier to flush than the isthmus end because the oviduct wall is much thicker.

10. Insert a 30 ga needle (attached to a 1 ml syringe containing 100 to 500 μl M2 medium (0.4% BSA) into oviducts with the aid of one pair of forceps. Once the needle is in place, overlay the forceps around the oviduct and needle to secure the oviduct.

11. Flush 100 to 200 μl of M2 medium through oviduct. If the flush is successful, the oviduct will distend and often a mass of debris can be seen exiting the oviduct.

12. If blastocyst stage embryos are desired, remove the entire tract by dissecting from the posterior part of the cervix to the ovaries. Dissect away the cervix and oviducts from all tracts. Flush each uterine horn with 500 to 700 μl M2 medium (0.4% BSA).

13. After flushing is completed, search for embryos using the inverted microscope at 10x magnification. Transfer embryos retrieved from flush into a culture dish with grids containing M16 medium (0.4% BSA) using a wiretrol micropipette.

14. Place 8 to 15 embryos in 5 to 7 μl drops of M16 medium (0.4% BSA). Rough estimations of medium volume can be made by the wiretrol micropipette (black

line on pipette = 5 μl). Place microdrops in 35 x 10 mm culture dishes without grids (5 to 7 drops/ dish). Cover drops with 3 to 4 ml oil and incubate at 37 C (5% CO_2/ 95% air; humid environment).

15. 2-cell embryos will develop into 8-cell embryos within 24 h, morulae within 30 to 36 h and blastocysts after 48 h of culture.

Preparation of Media

Sterile Saline

1. Prepare 100 ml saline by adding 0.9 g NaCl to 100 ml ddH_2O. Stir until NaCl is dissolved then sterile filter and store at room temperature until use.

M2 and M16 Media

1. M2 medium was developed by Quinn *et al.* (1982) and M16 medium was developed by Whittingham (1971).

2. Both M2 and M16 media can be purchased from Sigma in 1 l powder forms.

3. For M2 medium (M 5910), add powder to 900 ml of ddH_2O and stir until dissolved. Add 0.35 g Na bicarbonate and 4.35 g lactic acid (60% syrup), pH to 7.3 and bring up to 1 l with ddH_2O. Sterile filter and store at 4 C for 2 mo.

4. For M16 medium (M 1285), add powder to 900 ml of ddH_2O and stir until dissolved. Add 2.101 g Na bicarbonate and 4.35 g lactic acid (60% syrup), pH to 7.3 and bring up to 1 l with ddH_2O. Sterile filter and store at 4 C for 2 mo.

5. M2 and M16 may also be made from scratch. This procedure is taken from Hogan *et al.* (1986). Prepare the following stock solutions, sterile filter and store at 4 C. Stocks B and C can be used for up to 2 wk. Stocks A, D and E can be used for up to 2 mo.

Stock	Ingredient	Amount
Stock A (10x)	ddH_2O	100 ml
	NaCl	5.534 g
	KCl	0.356 g
	KH_2PO_4	0.162 g
	$MgSO_4+7H_2O$	0.293 g
	glucose	1.000 g
	Na lactate (60% syrup)	4.349 g
Stock B (10x)	ddH_2O	100 ml
	$NaHCO_3$	2.101 g
	Phenol Red	0.010 g
Stock C (100x)	ddH_2O	10 ml
	Na Pyruvate	0.036 g
Stock D (100x)	ddH_2O	10 ml
	$CaCl_2+2H_2O$	0.252 g
Stock E (10x)	ddH_2O	100 ml
	HEPES	5.958 g
	Phenol Red	0.010 g
pH to 7.0		

6. Make 100 ml of M2 and M16 media from stock solutions as follows:

Stock	M2 medium	M16 medium
	(ml)	(ml)
A	10.0	10.0
B	1.6	10.0
C	1.0	1.0
D	1.0	1.0
E	8.4	---
ddH_2O	77.0	77.0
Pen/Strep	1.0	1.0

7. Add 400 mg BSA (0.4% BSA) or 10 ml htFCS (10% htFCS) to 100 ml medium. Do not dissolve BSA by stirring, rather allow BSA to dissolve on its own. The BSA will dissolve within 15 minutes.

8. Adjust both media to pH 7.2 to 7.4 (ideally 7.35). Sterile filter media and store at 4 C for up to 1 wk.

Oil

1. Purchase embryo tested paraffin oil from Sigma (M 8410).

2. Paraffin oil (Baker oil; VWR; saybolt viscosity at 100 F, 345-355; catalog # S894-07) or silicon oil (Aldrich; # 14,615-3) can also be used but must be extracted before use. Generally, silicon oil is better for use in this system than paraffin oil.

3. Extract non-embryo tested oil by mixing with an equal volume of ddH_2O for 24 h. Repeat process a second time to ensure that toxins are extracted out of the oil. Sterile filter through a 0.45 μm filter and store at room temperature until use.

4. Oil can also be extracted immediately before use by mixing 30 ml oil with 20 ml ddH_2O in a 50 ml conical tube and incubating at 37 to 40 C for at least 20 min. Sterile filter through a 0.45 μm filter.

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BIOGRAPHICAL SKETCH

Alan Dale Ealy was born January 27, 1965, in Sharon, Pennsylvania, as the oldest son of Harold and Suzanne Ealy. He was reared on the family's Guernsey dairy farm in Sharpsville, Pennsylvania. He obtained his Bachelor of Science degree from Pennsylvania State University in 1987. During his studies at Penn State, he served as president of the Dairy Science Club, was a research assistant in two dairy science laboratories, was an intern in dairy promotions for the Pennsylvania Department of Agriculture, and was vice-president of Phi Sigma Kappa Fraternity. He was also honored in 1987 as the first runner-up for the American Dairy Science Association Undergraduate Oral Presentation.

After graduation, Mr. Ealy entered graduate school at Michigan State University under the supervision of Dr. R. L. Fogwell of the Department of Animal Sciences. His thesis research concerned effects of insulin and insulin-like growth factor I on corpus luteum function in cattle. He received his Master of Science in 1990. While at Michigan State, he married Monica Lynn Montalvo, and he has one stepson, William Lee.

Mr. Ealy began studies at the University of Florida in 1990, where he is now a candidate for the degree of Doctor of Philosophy. Under the supervision of Dr. Peter J. Hansen, Mr. Ealy investigated the acquisition of thermal resistance in mammalian embryos during early development. He was elected to membership in

Sigma Xi and Gamma Sigma Delta honorary societies and was selected as the 1994 recipient of the Sigma Xi Graduate Research Award.

Upon completion of the requirements for the degree of Doctor of Philosophy, Mr. Ealy will begin a postdoctoral fellowship with Dr. R. Michael Roberts at the University of Missouri. Here he plans to continue his studies on reproductive physiology of domestic animals.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Peter J. Hansen, Chairman
Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maarten Drost
Professor of Veterinary Medicine

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Professor of Anatomy and Cell Biology

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Frank A. Simmen
Associate Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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